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qPCRsoft

Software for
REAL-TIME PCR Thermal Cycler

Operating manual

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1 qPCRsoft Software

The qPCRsoft software can be used to create and perform PCR and Real-Time PCR experiments. This chapter describes the basic setup and the layout of the operating elements of the software.

Described software version

This description is based on the version qPCRsoft V 1.0.

Intended use

The qPCRsoft software serves for controlling the qTOWER and evaluating the data recorded with this device.

The manufacturer does not assume any liability for problems or damage caused by the unintended use of qPCRsoft.

qPCRsoft and the device to be controlled by it may only be operated by appropriately qualified and instructed personnel. The user must be familiar with the information given in this manual and in the user manual of the hardware.

1.1 How to use this manual

The following symbols and conventions are used to facilitate orientation in the manual:

→	Cross reference to other sections and/or figures.
Formatting	In the description of the operating procedures, menu commands, dialog boxes, buttons, options, etc. are highlighted in small capitals. Menu commands of a command sequence are separated by slashes (/), e.g. File / Open project. Buttons are additionally written in square brackets, e.g. [SAVE].

1.2 Installation of qPCRsoft

Administrator rights on the operating system are required for installing the program.

System requirements for installing qPCRsoft

For using qPCRsoft to control the Real-Time PCR device, your PC must fulfill the following minimum requirements:

Operating system:	Windows XP (SPS2) /VISTA/7
Processor	Pentium IV, > 1 GHz
RAM	1 GB
Available hard disk space	min. 300 MB
Interfaces	USB

Installation procedure

qPCRsoft is delivered on CD-Rom

1. Insert the CD in the CD-Rom drive. Normally, the installation's start window opens automatically.
   If this is not the case, start the "setup.exe" file on the CD.
   A selection dialog window for installing the device driver or the user management or for viewing the PDF files of the manuals appears.
2. Activate your language version.
3. Click on [INSTALL].
   The installation routine begins.
4. Follow the further instructions of the installation program.
5. Switch on the device at the power switch. Start qPCRsoft.

Note:
The software will only be installed correctly if it has been started once with administrator rights. A password for the program administrator must be entered during this first start.

1.3 Starting and exiting qPCRsoft

Starting qPCRsoft

1. To start qPCRsoft, click on the [START] button on the Windows desktop. Then go to the PROGRAMS folder and search for QPCRSOFT. Click on QPCRSOFT there.
2. Alternatively, you may click on the qPCRsoft icon on the Windows desktop. qPCRsoft is started.

If the user management has been installed, you will be prompted to enter user name and password. The qPCRsoft workspace will only become accessible if the entry of this data was successful.

Note:
An administrator and password must be defined during the first program start. Only the administrator can set up further user accounts or disable the user management.

Exiting qPCRsoft

File / Exit

1. To exit the qPCRsoft application, activate THE FILE / EXIT menu command.
2. The program will display a message if any projects that have not yet been saved are still open at this point.
3. If you want to save these projects, click on [YES]. Save the projects in the SAVE AS standard window
4. Then call up the FILE / EXIT menu command again to exit qPCRsoft.

1.4 The main window in qPCRsoft

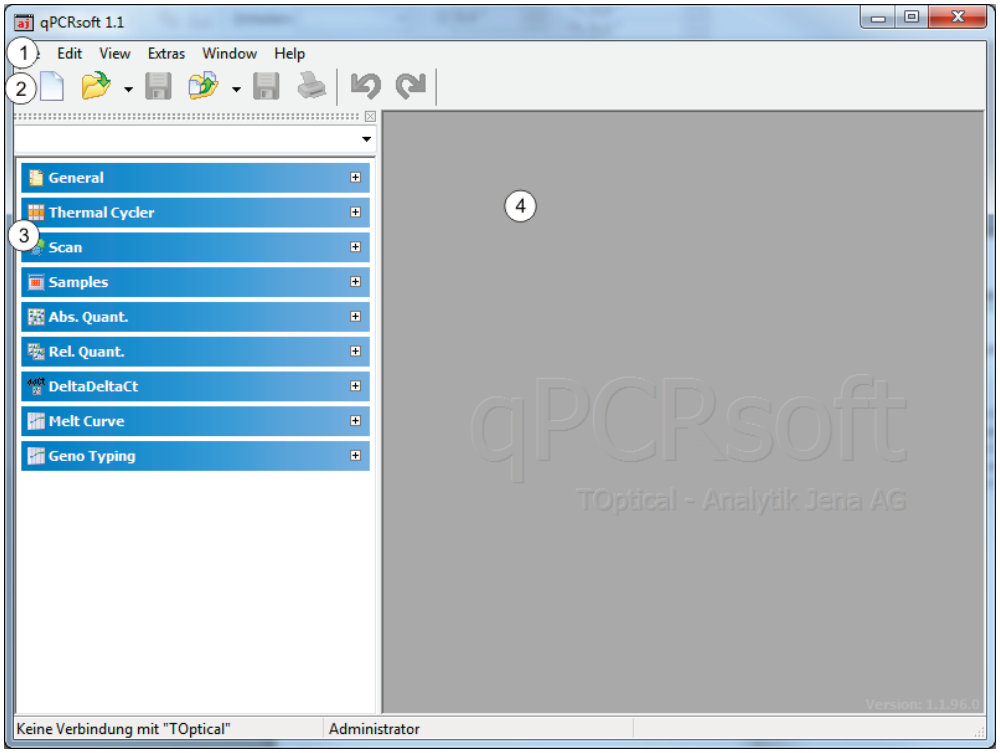


Fig. 1 Main window of the qPCRsoft software

After starting the qPCRsoft software the main window opens. It has the following sections:

Menu bar (1)	The menu bar contains the menu commands for, e.g., opening, editing and saving projects, managing user profiles, setting basic software options and a help function.
Toolbar (2)	Commands for editing projects are arranged in the toolbar. The commands offered in the toolbar may change according to context.
Project explorer (3)	In the project explorer a drop-down menu provides a quick overview of the most important information on the current open project.
Project interface (4)	The project interface is used to process projects. As soon as a new project is created or an existing project is loaded, a window opens in which all relevant settings for the respective project can be made.

1.4.1 Menu commands overview

The menu bar is context-sensitive and is automatically adapted to the program tasks. Menu items that are not necessary for the current work interface are automatically hidden. The following menu commands are available in the qPCRsoft software:

Menu	Function	Description
FILE	NEW	Opens a new project.
	OPEN TEMPLATE	Opens a template (RTSettings file (*.rts)).

	OPEN PROJECT	Opens a project (RTProject file (*.rtp)).
	OPEN AUTOM. SAVED PROJECT	Opens an automatically saved project.
	SAVE TEMPLATE	Saves a template file (RTSettings file (*.rts)) in the qPCRsoft standard folder.
	SAVE TEMPLATE AS	Saves a template file (RTSettings file (*.rts)) in any user-selected folder.
	SAVE PROJECT	Saves a project file (RTProject file (*.rtp)) in the qPCRsoft standard folder.
	SAVE PROJECT AS	Saves a project file (RTProject file (*.rtp)) in any user-selected folder.
	IMPORT ANALYSES	Opens an analysis file (RTAnalyses File (*.rta)).
	EXPORT ANALYSES	Saves an analysis file (RTAnalyses File (*.rta)).
	CLOSE	Closes a template or a project.
	CLOSE ALL	Closes all open projects or templates.
	PRINT	Prints a project.
	EXIT	Closes the software.
EDIT	UNDO	Reverses the last text modification (up to 10 steps).
	REDO	Restores the last deleted text item (up to 10 steps).
	CUT	Cuts a marked text area.
	COPY	Copies an active and/or marked text area.
	PASTE	Pastes atext area copied to the clipboard.
	DELETE	Deletes an active and/or marked text area.
	MARK ALL	Marks a complete text area.
	USER MANAGEMENT	Opens the window for creating user profiles and changing the password.
	SIGNATURES	Opens the window for managing digital signatures of projects.
VIEW	PROJECT EXPLORER	Switches the project explorer view in the main window on or off.
	TOOLBAR	Switches the toolbar view in the main window on or off.
SCAN	SET COLOR COMPENSATION	Opens the window for creating files for spectral color compensation.
EXTRAS	DEVICE INITIALIZATION	Resets the connected device to the initial state.
	DEVICE IDENTIFCATION	Activates the connected device
	EDIT COLOR MODULES	Opens the window for configuring the system with color filter modules.
	OPTIONS...	Opens the window for general basic software settings.
WINDOW	TILE HORZ	Arranges project windows horizontally.
	TILE VERT	Arranges project windows vertically.

	CASCADE	Arranges project windows in a cascaded fashion.
HELP	CONTENT	Opens the table of contents of the help function.
	INFO	Displays software information.

Menu	Function	Description
CYCLER	ADD EMPTY STEP	Adds a new step.
	DELETE STEP	Deletes a step.
	CUT STEP	Cuts a step and copies it to the clipboard.
	COPY STEP	Copies the parameters in one step into the clipboard.
	PASTE STEP	Inserts a copied step.
SCAN	EDIT COLOR COMPENSATION	Opens the window for creating files for spectral color compensation.
SAMPLES	EDIT LAYOUT	Edit sample table.
	COPY LAYOUT	Copy the area of the sample table.
	PASTE LAYOUT	Insert the copied area of the sample table.
	PREVIEW LAYOUT	Detailed view of the plate assignment
MONITORING	START QPCR RUN	Start the PCR run.
	STOP QPCR RUN	Stop the PCR run.
	PAUSE QPCR RUN	Pause the PCR run.
	DISPLAY OPTIONS	Display options for the product accumulation curves
ABSQUANT	ADD ABS. QUANTIFICATION	Create new evaluation.
	DELTE ABS. QUANTIFICATION	Delete evaluation.
	OPTIONSABS. QUANTIFICATIONG	Opens a window for basic evaluation settings.
	AUTOMA. THRESHOLD	Automatic determination of the fluorescence threshold for detecting Ct values.
	IMPORT STANDARD CURVE	Import a saved standard curve.
RELQUANT	ADD REL. QUANTIFICATION	Create new evaluation.
	DELTE REL. QUANTIFICATION	Delete evaluation.
	OPTIONSREL. QUANTIFICATIONG	Opens a window for basic evaluation settings.
	AUTOMA. THRESHOLD	Automatic determination of the fluorescence threshold for detecting Ct values.
	IMPORT STANDARD CURVE	Import a saved standard curve.

DELTADELTACT	ADD ΔΔCT QUANTIFICATION	Create new evaluation.
	DELTE ΔΔCT. QUANTIFICATION	Delete evaluation.
	OPTIONSΔΔCT. QUANTIFICATIONG	Opens a window for basic evaluation settings.
	AUTOMA. THRESHOLD	Automatic determination of the fluorescence threshold for detecting Ct values.
MELTING CURVE	ADD MELTING CURVE	Create new evaluation.
	DELTE MELTING CURVE	Delete evaluation.
	OPTIONSMELTING CURVE	Opens a window for basic evaluation settings.
	AUTOMA. THRESHOLD	Determine threshold automatically.
GENOTYPIN	ADD GENOTYPING	Create new evaluation.
	DELETE GENOTYPING	Delete evaluation.
	OPTIONS GENOTYPING	Opens a window for basic evaluation settings.
	AUTOM. THRESHOLD	Automatic determination of the fluorescence threshold for detecting Ct values.

1.4.2 Overview of the tools in the toolbar

The buttons in the toolbar are mostly context-sensitive. The program automatically adjusts the toolbar to the window content and adds buttons provided that this is required and useful for the current project window view. Buttons not accessible for the current contents of the workspace are hidden.

View / toolbar

You can display or hide the toolbar via the VIEW / TOOLBAR menu command.

Button	Command	Function
General		
	NEW	Opens a new project.
	OPEN TEMPLATE	Opens a template.
	SAVE TEMPLATE	Saves a template.
	OPEN PROJECT	Opens a project.
	SAVE PROJECT	Saves a project.
	PRINT PROJECT	Prints a project.
	UNDO	Reverses the last modification.
	REDO	Restores the last deleted modification.
	CUT	Cuts a marked area.

	COPY	Copies an active and/or marked area.
	PASTE	Pastes an area copied into the clipboard.
	DELETE	Deletes an active and/or marked area.
PCR protocol		
	ADD EMPTY STEP	Adds a new step.
	DELETE STEP	Deletes a step.
	ACTIVATE MELTING CURVE	Adds a step for melting curve determination.
	CUT STEP	Cuts a step and copies it to the clipboard.
	COPY STEP	Copies the parameters in one step into the clipboard.
	PASTE STEP	Pastes a copied step.
Color		
	EDIT COLOR COMPENSATION	Opens the window for creating files for spectral color compensation.
Samples		
	EDIT LAYOUT	Assigns changes that were made to the sample table.
	COPY LAYOUT	Copies an area in the sample table.
	PASTE LAYOUT	Pastes a copied area from the sample table.
	PREVIEW LAYOUT	Displays a complete view of the plate assignment.
Monitoring		
	START PCR PROTOCOL	Starts the PCR run.
	STOP PCR PROTOCOL	Ends the PCR run.
	OPTIONS	Display options of the product accumulation curves.
Evaluation/absolute quantification		
	NEW	Creates a new evaluation
	DELETE	Deletes an evaluation
	OPTIONS	Opens a window for basic evaluation settings.
	AUTOMATIC THRESHOLD	Automatic determination of the fluorescence threshold value for Ct value determination.
	IMPORT STANDARD CURVE	Imports a saved standard curve.
Evaluation/relative quantification		
	NEW	Creates a new evaluation.
	DELETE	Deletes an evaluation.

	OPTIONS	Opens a window for basic evaluation settings.
	AUTOMATIC THRESHOLD	Automatic determination of the fluorescence threshold value for Ct value determination.
	IMPORT STANDARD CURVE	Imports a saved standard curve.
Evaluation/ΔΔCt analysis		
	NEW	Creates a new evaluation.
	DELETE	Deletes an evaluation.
	OPTIONS	Opens a window for basic evaluation settings.
	AUTOMATIC THRESHOLD	Automatic determination of the fluorescence threshold value for Ct value determination
Evaluation/melting curve		
	NEW	Creates a new evaluation.
	DELETE	Deletes the current evaluation.
	OPTIONS	Opens a window for basic settings for the evaluation.
	AUTOMATIC THRESHOLD	Automatically determines the threshold.
Evaluation/Genotyping		
	NEW	Creates a new evaluation.
	DELETE	Deletes the current evaluation.
	OPTIONS	Opens a window for basic settings for the evaluation.
	AUTOMATIC THRESHOLD	Automatically determines the threshold.

1.4.3 Project explorer components

Different menus (2) in the project explorer offer a quick overview of the currently processed project. Individual projects can be selected via a selection list (1). The information on the individual menus can be displayed or hidden via the [+] and [-] (3) buttons.

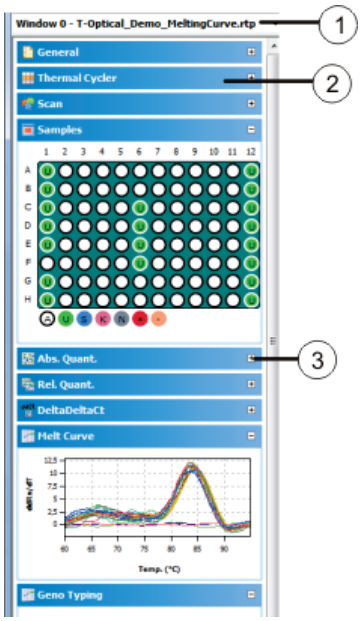


Fig.2 Organization of the project explorer

Menu	Information
GENERAL	Project title, user, date, timeand device
THERMOCYCLER	Graphic display of the history of the PCR program in the active project
SCAN	Overview of the colors and areas of the PCR plate that are being scanned
SAMPLES	Displays a short info text on the plate layout. The edit mode for the plate layout displays detailed information on the selected well.
ABSOLUTE QUANTIFICATION	Graphic display Ct against log concentration
RELATIVE QUANTIFICATION	Graphic display Ct against log concentration
ΔΔCT	Graphical display dCt(V) compared to log concentration
MELTCURVE	Graphical display of melting curve compared to temperature
GENOTYPING	Graphical display of dRn wild type compared to dRn mutant as a scatter plot or bar graph

View / Project explorer

You can display or hide the project explorer via the VIEW / PROJECT EXPLORER menu command.

1.4.4 Project interface with project window

The project interface is initially empty when starting the program. Only when a new project is created or a saved project and/or a template is loaded the project window opens.

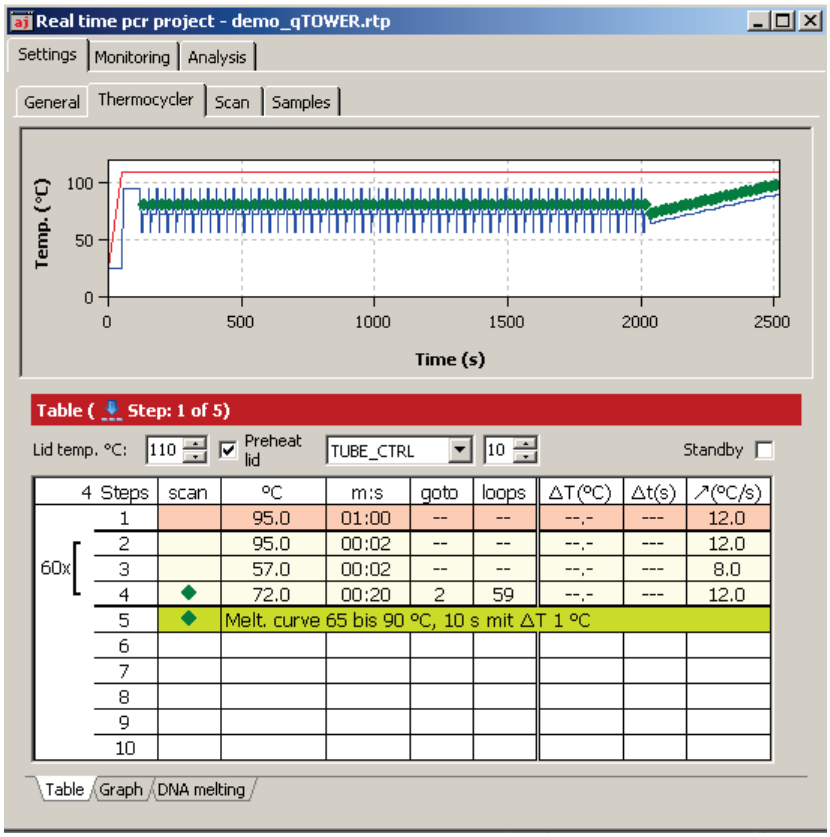


Fig. 3 View of a project window

In the project window all parameters, measuring data and evaluations for one PCR plate are combined. The basic functions are arranged on the three main tabs on the top:

Tab	Function
SETTINGS	Contains all functions required for defining Real-Time PCR runs.
MONITORING	Contains different tools for monitoring Real-Time PCR runs.
EVALUATION	Includes the evaluation algorithms implemented in the software for analyzing acquired data.

These three tabs are always visible. The view of the project window changes depending on the selection of a function tab. To indicate the tab the descriptions refer to, the tabs and list sheets are listed in the order in which they were activated and divided by a slash (similar to a menu command), e.g. SETTINGS / THERMOCYCLER / TABLE.

2 Managing projects

The qPCRsoft software saves all experiments in the project files. A project contains different information required to perform a Real-Time PCR experiment:

- Description of the experiment
- PCR protocol
- Scan settings of the optical system
- Plate assignment with detailed information on each sample
- Measuring results and the corresponding evaluation after the experiment has been performed

2.1 Creating a new project or opening a project

A project is always indicated in a project window in a section of the project interface of the main window.

Creating a new project

File / New

To create a new project, click on or call up the FILE / NEW menu command.

A new project with standard presets is created in the project window.

Creating a new project based on a template

A new project can be opened with saved presets (template):

File / Open template...

1. Click on or call up the FILE / OPEN TEMPLATE... menu command.
2. In the standard window, select the desired template to open files (RTSettings file (*.rts)) and confirm the selection with [OK].

A new project with the parameter settings of the template is created in the project window.

Opening a saved project

File / Open project...

1. Click on or call up the FILE / OPEN PROJECT... menu command.
2. In the standard window, select the desired project to open files (RTProject file (*.rtp)) and confirm the selection with [OK].

The project with the parameter settings, measurement results and evaluations is created in the project window.

Opening an automatically saved project

File / Open autom. saved project

The qPCRsoft programs enables you to automatically save the last completed Real-Time PCR run to a folder of your choice and thus prevents data loss due to unexpected terminations of a PCR run. The terminated measurement can be recovered with the FILE / OPEN AUTOM. SAVED PROJECT menu command and saved under a different name as a project file.

To change the storage location of the file, proceed as follows:

1. Select EXTRAS / OPTIONS to open the window of the same name.
2. Open the GENERAL tab.
3. Click […] and select a storage location.

The AUTOM. SAVE option enables you to switch the automatic save function on and off.

Viewing the projects

Window / ...

You can open several projects at a time. Each project is displayed in its own project window. With the commands from the WINDOW menu, the project windows can be arranged horizontally, vertically or overlapping.

2.2 Saving a template

All basic information required for performing an experiment that is stored in the project window on the SETTINGS tab, such as the description of the experiment, the PCR protocol, scan settings of the optical system and the plate assignment, can be saved as a template.

File / Save template as...

1. Call up the FILE / SAVE TEMPLATE AS command.
2. To save files enter the name of the template in the standard window and save the template with [OK].

Templates are saved with the extension "*.rts".

File / Save template

The changes in a template can be saved with the FILE / SAVE TEMPLATE menu command. Optionally, you can click on the symbol in the toolbar.

2.3 Saving a project

You can save the project with all parameters of the PCR run, the fluorescence curves and evaluations.

File / Save project as...

1. Call up the FILE / SAVE PROJECT AS... command.
2. To save files enter the name of the template in the standard window and save the template with [OK].

Projects are saved with the extension "*.rtp".

File / Save project

The changes in a project can be saved with the FILE / SAVE PROJECT menu command.Optionally, you can click on the symbol in the toolbar.

2.4 Importing/exporting analyses

Settings for the data evaluations of a project can be saved (exported) and later transferred (imported) to an open project. The evaluations are applied to the open project when they are imported.

File / Export analyses

1. Select the FILE / EXPORT ANALYSES menu command.
2. To save files enter the name of the analysis and save the files with [OK].

Analyses are saved with the extension "*.rta".

File / Import analyses

1. Select the FILE / IMPORT ANALYSES menu command.
2. Select the name of the analysis in the default window for opening files and import the analysis into the current project by clicking [OK].

2.5 Closing project windows

File / Close

File / Close all

The FILE / CLOSE menu command closes the active project window. To close all project windows, select the FILE / CLOSE ALL menu command. If any unsaved changes have been made in project windows, a confirmation prompt appears.

2.6 Printing

You can specify the desired contents for printing a project in a selection list:

File / Print

1. Select the FILE / PRINT menu command.
2. Manage the print output using the displayed lists. Select the desired information in the list on the left and click to transfer it to the print list on the right. To remove undesired information from the print list, click .
3. Click [PRINT] to start the printout.

Select [OPTIONS] to configure the printout, or [PREVIEW] to display a page view of the print image.

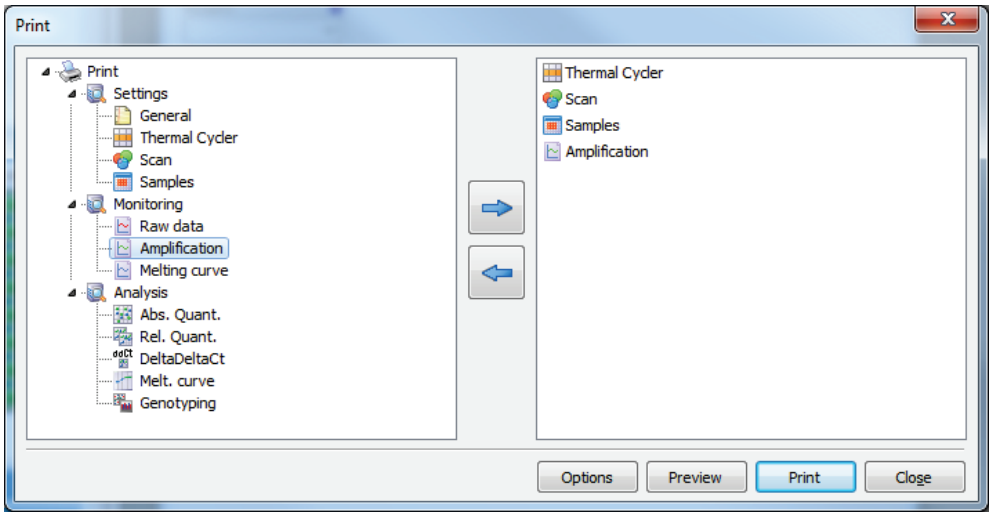


Fig. 4 PRINT window

The individual print modules are sorted into the SETTINGS, MONITORING and ANALYSIS subgroups in the PRINT window.

2.7 Signing projects

Projects can be signed if the user management has been activated.

Edit / Signatures

1. Activate the project you would like to sign.
2. Select the EDIT / SIGNATURES menu command to open the SIGNATURE window.
3. Click [LOGIN] and log in with your USER NAME and PASSWORD.
4. Select a status from the list: DOCUMENT SEEN, DOCUMENT APPROVED, DOCUMENT IN PROGRESS or DOCUMENT REJECTED.
5. Confirm with [OK].

3 Settings for a Real-Time PCR experiment

If you want to start a new project, create a new project or open a template:

- ❑ Create an empty project with in the toolbar.
- ❑ Optionally, open a template with to use the previously stored parameters for the new project or to modify them.

All functions necessary to create a new project are combined under the SETTINGS tab. Additional tabs on the second level are assigned to the SETTINGS tab:

Tab page	Function
GENERAL	Allows you to enter general information and remarks
THERMOCYCLER	Used for programming PCR protocols
SCAN	Definition of the colors to be measured and settings for the measuring parameters
SAMPLES	Opens the sample table in which detailed information on each sample can be saved and groups of experiments defined

3.1 Entering general information on the project

Settings / General project window

You can save general information on each project. The entries can be made on the GENERAL tab:

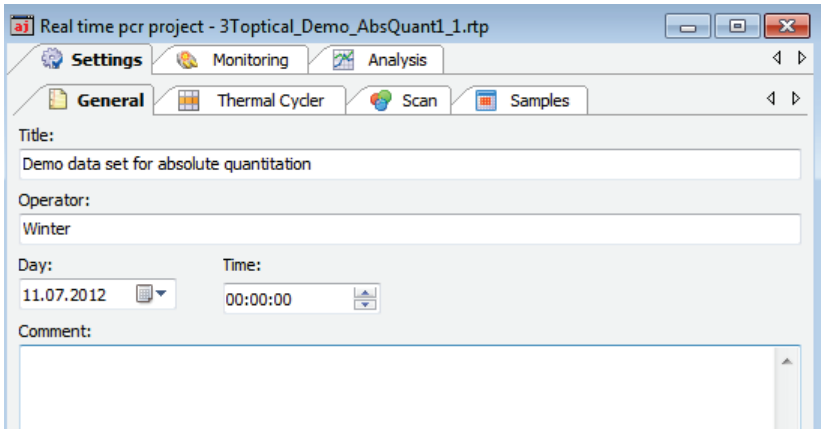


Fig.5 Real-time PCR run – General information

Command	Description
TITLE	Analysis title
OPERATOR	User If you are using a user management option, the user name you signed in with will be entered automatically.
DAY / TIME	Date the project was created
COMMENT	Comment input

Note:

Edit /…

For text inputs on the tab GENERAL being able to use the usual commands for copying, cut out and insert of texts. These commands are arranged in the menu EDIT.

3.2 Creating aPCR protocol

Settings / Thermocycler project window

A PCR protocol must be programmed for each Real-Time PCR experiment. All necessary functions are combined in the SETTINGS / THERMOCYCLER project window. The screen is divided in three sections: graphical program preview (1), program header (2) and program table (3).

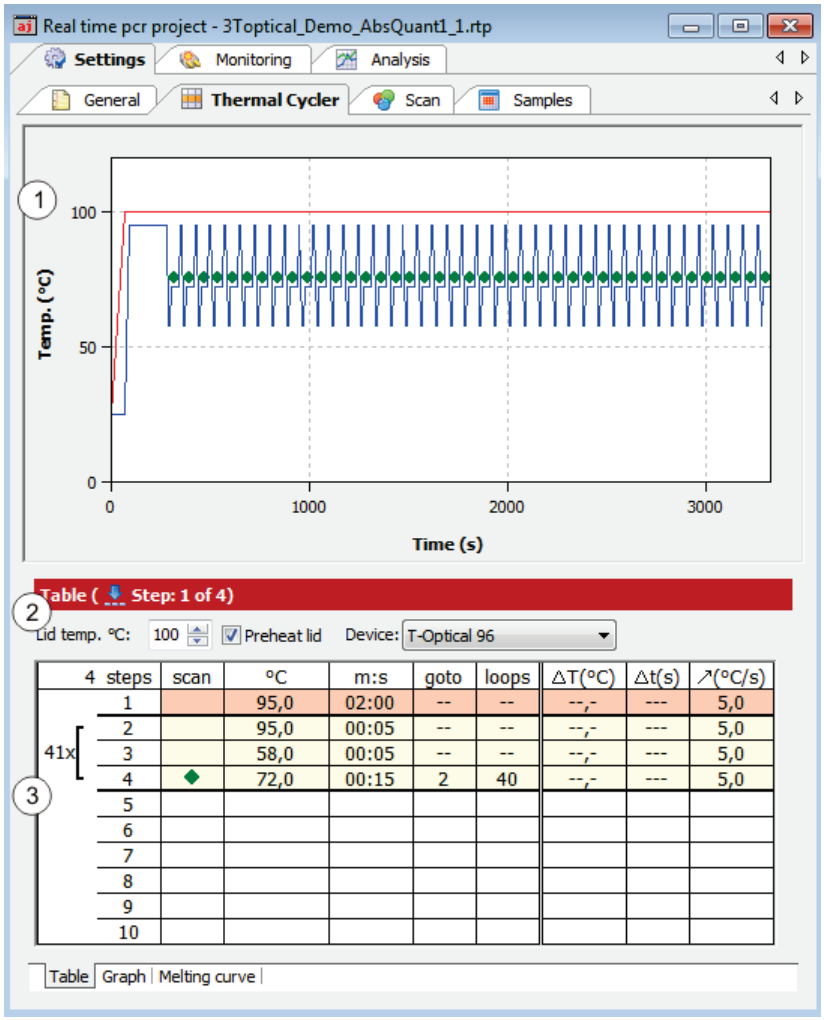


Fig. 6 Program table for creating PCR protocols

The program preview illustrates the history of the PCR protocol. The program header defines the general conditions for the PCR protocol, such as the programmed lid temperature, the lid heating mode and the thermocycler's block type. The programming table offers a clear representation of the individual steps of the program.

The THERMOCYCLER tab contains three list sheets. The tabs for switching between the list sheets are located at the bottom of the window.

List sheet	Function
TABLE	Contains a table for programming PCR programs.
GRAPH	Offers the option for graphical programming of PCR programs.
MELTING CURVE	Is used to enter parameters for measuring a melting curve.

3.2.1 Editing a PCR protocol

Programs can be edited in the tabular or graphical representation.

You can switch between the two alternative representations via the TABLE and GRAPH screens. To edit one step (the current active step is highlighted light red) you can use the corresponding function from the CYCLER menu in the menu bar or the corresponding symbol from the toolbar:

Symbol	(CYCLER/...) menu command	Description
	ADD EMPTY STEP	Inserts a new step behind the active step
	DELETE STEP	Deletes the active step
	CUT STEP	Cuts the active step
	COPY STEP	Copies the active step
	PASTE STEP	Inserts a copied step behind the active step

3.2.2 Entering information in the program header

The program header contains several general, device specific program settings:

- Options for lid heating
- Temperature control method
- Stand-by mode activation after the PCR is complete

Fig. 7 Program header of the PCR program (example for qTower settings)

Option/List	Description
LID TEMP.	Sets the lid temperature. The temperature of the heating lid should generally be slightly above the maximum block temperature to prevent liquids from evaporating from the reaction mixture and condensing at the walls or lid of the reaction cups.
PREHEAT LID	If activated, this option preheats the lid to the set LID TEMPERATURE before the actual PCR program starts. This is the recommended standard setting to ensure the formation of a homogeneously tempered air cushion between the sample containers. This leads to an improved temperature uniformity between the samples. While the lid is being heated, the block is kept at a constant 25°C. If the option is deactivated, the PCR program starts while the lid is still being heated. Adjustable lid temperature: 30 – 110 °C **Note:** After the heating lid has reached the target temperature, this is followed by a 40-second equilibration before the program is started.

List: temperature regulation of the thermoblock (for qTower)	BLOCK_CTRL: The block temperature is regulated according to the selected temperature program. Particularly if the heating and cooling rates are high and the hold times are short the actual sample temperature can differ from the desired temperature. TUBE_CTRL: The sample temperature is calculated upfront based on the measured block temperature and is then regulated to that temperature. This method is particularly recommended for fast protocols. MAN_CTRL: The manual block temperature control allows a very accurate matching of the experiment to the device thanks to the factor that can be adjusted between 0 and 10. The method for factor 0 is BLOCK_CTRL and for factor 10 TUBE_CTRL.
Select block type (for TOptical)	The installed block module is automatically detected by the TOptical thermocycler during device initialization (with/without gradient function). When creating a new program this module is accepted as default. However, it is possible to create a program for a different block format than that currently installed (for example, a program with a temperature gradient although no block capable of a gradient has currently been inserted). The desired block type can be selected from the list.
Device selection (for qTower 2.0 and 2.2)	The installed qTOWER (with (2.2)/without gradient function (2.0)) is automatically detected during initialization. When creating a new program this type is accepted as default. However, it is possible to create a program for a different device type than that currently installed (for example, a program with a temperature gradient although no block capable of a gradient has currently been inserted). To do so, select the type from the devices list.

3.2.3 Entering the PCR protocol

The PCR program is entered into the program table of the TABLE list sheet. One row of the table contains the parameters of a temperature step.

You can navigate in the program table by using the mouse or the four arrow keys ([←] [→] [↑] [↓]) on the keyboard. Each entry is confirmed with the [ENTER] key or the [→] arrow key. The cursor jumps into the corresponding field in the adjoining column. If the cursor is in the last row, an additional temperature step is inserted with [↓].

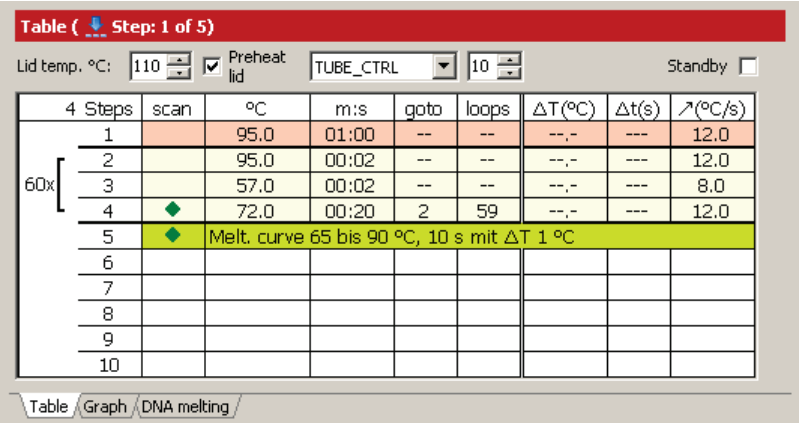


Fig.8 Program table for a PCR

The following values are entered into the table or calculated from the default values:

Value	Description
SCAN	Measures the sample fluorescence during this step, if marked
STEPS	Number of the step in the temperature program Is automatically numbered consecutively
°C	Enter the target temperature of the step in °C
M:S	Enter the hold time of the target temperature
GOTO / LOOP	Define the loop with the number of repetitions for a cycle
ΔT(°C)	Enter the increment or decrement of the target temperature within the PCR run
ΔT(S)	Enter the increment or decrement of the hold time within the PCR run
↗(°C/S)	Enter the heating or cooling rate to reach the target temperature in the temperature step

Inserting a new temperature step / deleting a temperature step

Cycler / Add empty step

- ❑ You can insert an additional temperature step to the program table with the **[↓]** arrow key.

  Optionally, you can add another temperature step with the CYCLER / ADD EMPTY STEP menu command or .

  The total number of steps and the current processed step are displayed in the protocol header.

Cycler / Delete step

- ❑ To delete a program step move the cursor to the program row and click on .

  Optionally, you can call up the CYCLER / DELETE STEP menu command.

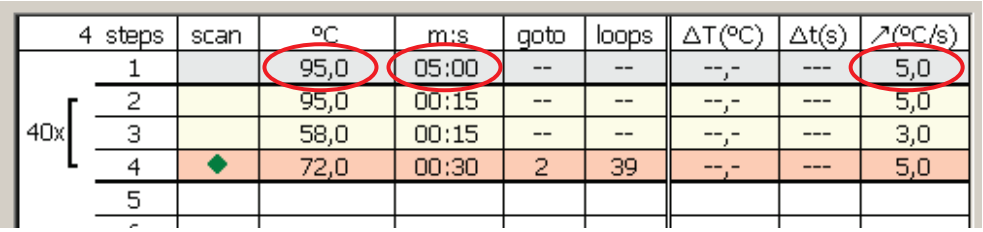
Entering the target temperature, hold time and heating/cooling rates

- ❑ Enter the target temperature of the temperature step into the **°C** column.
- ❑ Into the **m:s** column, enter the hold time in the format "minutes:seconds" (e.g. hold time of a duration of 1 min 20 s: 1:20).

- For special applications or the transfer of protocols from other devices it may be required to adjust the ***heating and cooling rates***.
  Enter the average heating and cooling rate for each individual step in the ↗**(°C/s)** column.

  Note:
  The value in the ↗**(°C/s)** column defines the speed at which the target temperature is reached. If the temperature is be heated (or cooled) at a speed of 3°C per second between step 2 and step 3 the value 3.0 must be entered for step 3.

  Note:
  If the speed is to be reduced for the whole program the heating or cooling rates of all steps must be adjusted. The settings are only valid for that one program.



	4 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
	1		95,0	05:00	--	--	--,-	---	5,0
40x	2		95,0	00:15	--	--	--,-	---	5,0
	3		58,0	00:15	--	--	--,-	---	3,0
	4	◆	72,0	00:30	2	39	--,-	---	5,0
	5								

Fig. 9 Entering the target temperature, hold time and heating/cooling rates in the PCR table

Defining loops

Program sequences that are repeated regularly can be summarized in ***loops***. Generally, a loop is then defined by a target step for the return ("GOTO" and the number or repetitions ("LOOPS").

	3 Steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
	1		95.0	05:00	--	--	--,-	---	5.0
40x	2		95.0	00:30	--	--	--,-	---	5.0
	3		68.0	00:30	2	39	--,-	---	5.0
	4								

Fig. 10 Programming a loop in the PCR table

1. Place the cursor on the last step of the future loop (step 4 in the example above).
2. Enter the number of the target step into the GOTO column ("2" in the example above).
3. In the LOOPS column enter the number of repetitions ("39" in the example above).

After entering the target step and the repetitions the programmed loop is displayed as a bracket on the left side of the table (see figure above).

Note:
The total number of loops displayed in the bracket is determined from the number of programmed repetitions plus 1, as the corresponding sequence of steps prior to reaching the loop has already cycled once.

Entering increments/decrements for temperature and hold time

By programming ***increments/decrements*** the temperature or hold time can be modified by a specific amount from one cycle to the other within a loop.This technology is for instance used for the "Touch Down" PCR.

3 steps		scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
40x[	1		95,0	05:00	--	--	--,-	---	5,0
	2		95,0	00:30	--	--	--,-	---	5,0
	3		68,0	00:30	2	39	-1,0	5	5,0

Fig. 11 Entering increments for temperature and hold time

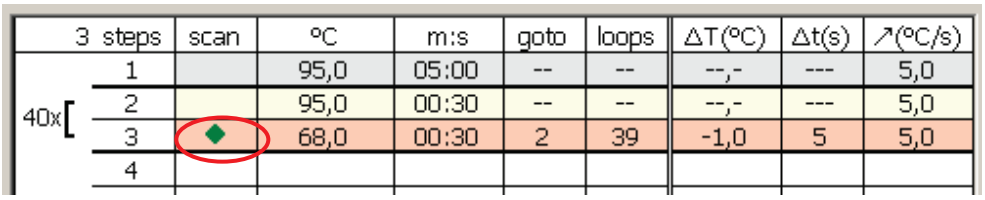
- ❑ Enter the desired changes for the temperature step the values of which you wish to modify within the loop. Use the (-) sign to specify a decrement, i.e. temperature or hold time are reduced from cycle to cycle by the specified amount. No sign or (+) mark a decrement with the result that the parameter increases by the specified amount from cycle to cycle.
- ❑ To modify the target temperature in steps, enter the changes in the ΔT(°C) column.
- ❑ To modify the hold temperature in steps, enter the changes in the ΔT(S) column.

Note:
The modified step must be within a loop. Otherwise, the entries in the columns ΔT(°C) and ΔT(S) have no effect.

Note:
The extension of the hold time of one step affects the total run time of a protocol. A program with many cycles and significant hold time increases takes substantially longer than a comparable program without a programmed extension.

Arranging a fluorescence measurement

- ❑ To define the measurement of the sample fluorescence in a temperature step of the PCR protocol, click on the SCAN column of the temperature step. A green diamond (◆) indicates that the measurements is active.



3 steps		scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
40x[	1		95,0	05:00	--	--	--,-	---	5,0
	2		95,0	00:30	--	--	--,-	---	5,0
	3	◆	68,0	00:30	2	39	-1,0	5	5,0
	4								

Fig. 12 Defining a fluorescence measurement in the PCR run

Define the parameters for the fluorescence measurement on the SCAN tab (→ see section "Defining the parameters for the fluorescence measurement" p. 30).

Note:
If a step for melting curve determination is added, the scanning process is automatically activated for this step. For all other steps of the PCR protocol the allocation must be made manually.

Adding a melting curve

Settings / Thermocycler / DNA melting project window

For experiments with intercalating dyes we recommend to check the specificity of the products by measuring a melting curve. The device can be programmed to add the corresponding step in the PCR protocol. Activate the option ACTIVE on the MELTING CURVE tab.

	4 Steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
	1		95.0	01:00	--	--	--.-	---	12.0
60x	2		95.0	00:02	--	--	--.-	---	12.0
	3		57.0	00:02	--	--	--.-	---	8.0
	4	◆	72.0	00:20	2	59	--.-	---	12.0
	5	◆	Melt. curve 65 bis 90 °C, 10 s mit ΔT 1 °C						
	6								
	7								
	8								
	9								
	10								

Fig.13 PCR protocol with a step for determining the melting curve

- ❑ A melting curve can be added to the program table by activating the option ACTIVE on MELTING CURVE tab.

The melting curve is added to the last temperature step in the table.

- ❑ To remove a melting curve from the program table deactivate the option ACTIVE on melting curve tab.
- ❑ Set the individual parameters of the melting curve step on the MELTINGCURVE tab:

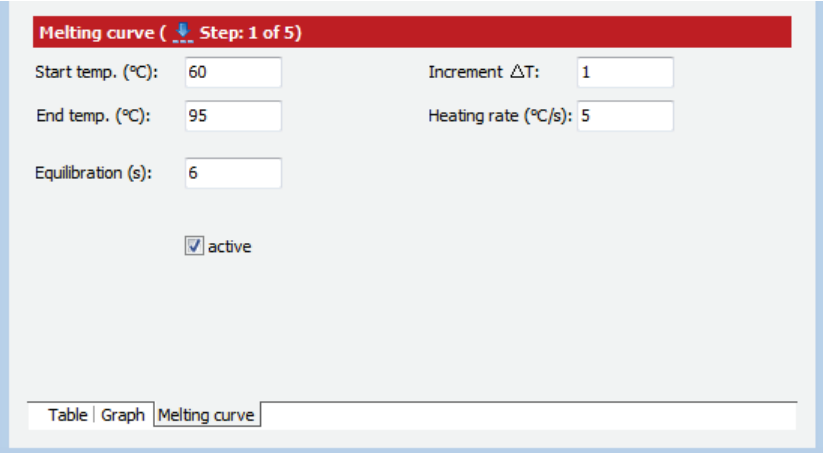


Fig.14 DNA melting curve screen

The following parameters can be modified:

Parameter	Description
START TEMP. (°C)	Start temperature of the melting curve
END TEMP. (°C)	End temperature of the melting curve
EQUILIBRATION (S)	Time for equilibration of the sample in a temperature before a measurement is performed
INCREMENT ΔT	Difference between two adjoining temperature steps in °C
HEATING RATE (°C/S)	Heating rate of the block
ACTIVE	Add a melting curve on the end of the PCR protocol.

The fluorescence measurement is automatically defined when the melting curve is recorded.

Programming the temperature gradient (TOptical 96 Gradient and qTOWER 2.2 only)

Gradients can be programmed over the whole temperature range of the thermoblock between 3.0 °C and 99 °C. The gradient range can be max. 40 °C.

- ❑ Gradients are defined in the program table by entering two temperature values separated by a dash. The first value entered corresponds to the temperature in column 01 (left block side), the second value to the temperature in column 12 (right block side).

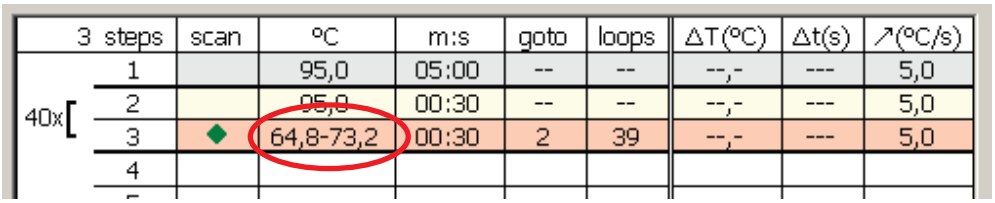


Fig.15 Entering the temperature gradient

The progression of the temperature gradient can be reviewed on the GRADIENT tab. The temperatures in the individual columns of the block are summarized in a table below the bar graph.

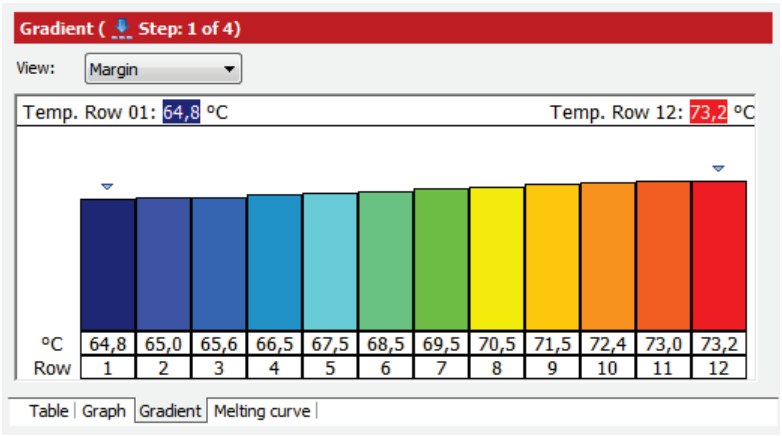


Fig.16 Gradient tab with MARGIN gradient function

- ❑ Adjust the gradient progression by entering temperature values for the first (TEMP. ROW 01) and last (TEMP. ROW 12) column of the block.

  The values displayed in the table are then updated.

- ❑ Alternatively you can click on the top end of column 1 or column 12 and change the height of the column with the left mouse button depressed. Moving the columns changes the respective temperature value accordingly.

Programming a linear gradient (TOptical 96 Gradient and qTOWER 2.2 only)

In addition to the option of defining a gradient by entering temperature values for columns 1 and 12, the gradient can also be programmed starting with an annealing temperature in the center of the block using fixed temperature increments.

- ❑ On the GRADIENT tab, select the LINEAR option from the VIEW list.
- ❑ Enter the temperature for column 6 in the ANNEALING TEMP. field and the desired temperature change from column to column in the INCREMENT field.

  The values displayed in the table are updated after the values have been entered.

- Another option for adjusting the gradient is to click on the top end of column 6 and change the height of the column with the left mouse button depressed. Moving the column the changes the annealing temperature accordingly.

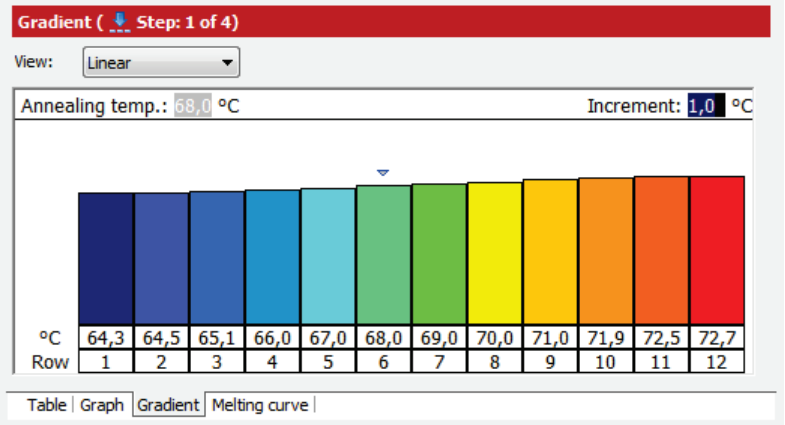

Gradient (Step: 1 of 4)
View:
Linear
Annealing temp.: 68,0 °C
Increment: 1,0 °C
°C 64,3 64,5 65,1 66,0 67,0 68,0 69,0 70,0 71,0 71,9 72,5 72,7
Row 1 2 3 4 5 6 7 8 9 10 11 12
Table
Graph
Gradient
Melting curve

Fig.17 Programming a linear gradient

3.2.4 Graphical display and programming the PCR protocols

Settings / Thermocycler / Graph project window

In the SETTINGS / THERMOCYCLER project window, the history of a programmed PCR protocol is displayed graphically in the TABLE and GRAPH screens. It represents the temperature curve of the block (blue line) and the heating lid (red line) over time. The green diamond symbolizes steps during which the fluorescence is measured.

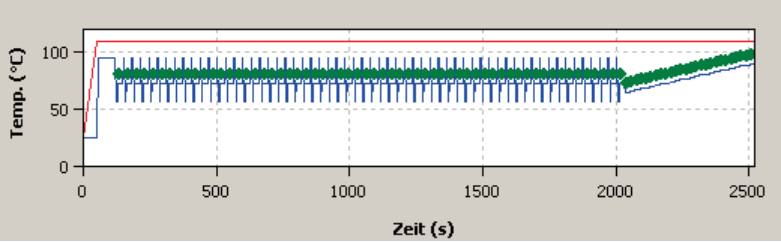


Fig.18 Graphical representation of a PCR protocol

Programs are generally created in the table view, which allows new steps to be programmed quickly and provides a summarized overall survey of the protocol structure. Some programming options are only available in the table view. The graphical programming mode additionally offers a schematic representation of the temperature profile and the option to adjust protocols quickly. In the SETTINGS / THERMOCYCLER project window you can switch between the table view and the graphical mode via the TABLE or GRAPH sheets.

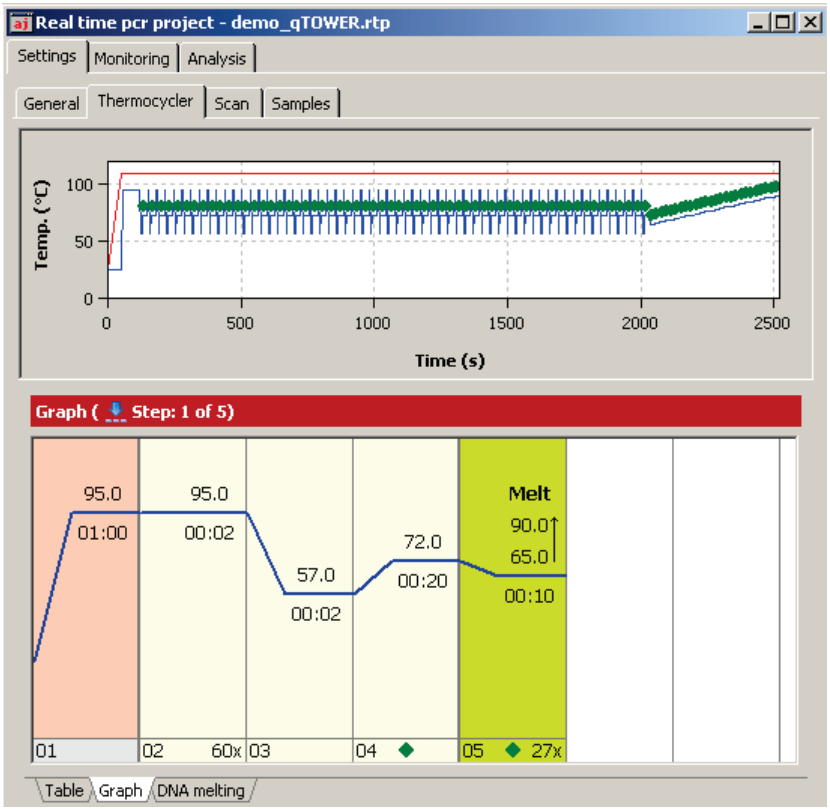


Fig.19 Graphical programming mode

The graphical programming is generally performed in the same way as in the programming table.

- ❑ By selecting a step (clicking on it) it becomes active and is highlighted light red.
- ❑ The bottom part of the display shows the number of the corresponding protocol step.
  Next to it, the number of repetitions in loops (right) and scanning processes (middle) can be programmed in this field.
  The number of repetitions is indicated as a figure (e.g. 40x) and can be edited by clicking on it with the left mouse button.
  Planned measurements are displayed by means of a green diamond in the middle of the field and can also be selected or deselected with the left mouse button.
- ❑ Temperatures and hold times are indicated as numerical values above or below the blue line that displays the corresponding temperature level at the individual steps. The values can be modified by clicking with the left mouse button. Melting curve steps are marked with the addition MELT. Additionally, a symbol of an arrow pointing upwards is displayed with the melting curve steps (↑).

  Note:
  The number of repetitions in melting curve steps cannot be modified.
- ❑ The temperature value at each step can be modified using the mouse. For this purpose, press and hold the left mouse button to move the blue line of the temperature curve up or down.

3.3 Defining the parameters for the fluorescence measurement

Settings / Scan project window

The product accumulation is measured by means of the increase in fluorescence in the Real-Time PCR. The following measurement parameters must be defined for that purpose:

- Dyes to be measured
- Temperature step of the PCR protocol during which a measurement is to take place
- The area on the PCR plate that is to be scanned

The colors to be measured are defined in the SETTINGS / SCAN project window:

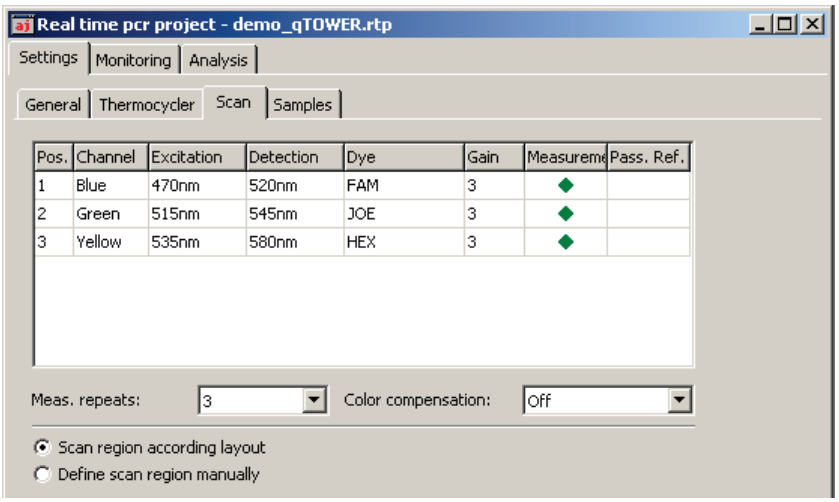


Fig.20 Project window with settings for the fluorescence measurement

The SCAN tab contains a table with different parameters for defining the scan properties:

Up to four (qTOWER) or six (TOptical, qTOWER 2.0/2.2) color channels with different excitation and detection wavelengths can be used for the fluorescence measurement.The parameters of the fluorescence measurement apply for all layout samples on which a measurement is to be performed.

Parameter / Option	Description
POS.	Color module position in the device
CHANNEL	Color channel description
EXCITATION / DETECTION	Excitation and detection wavelength of the color channel
DYE	Define the dye to be measured for the corresponding channel in the table by means of a selection list
GAIN	Regulation of signal intensity The intensity can be adjusted in steps between 0 and 10. The higher the value, the higher the fluorescence signal in the corresponding channel. Standard value: 3
MEASUREMENT	Activates dye measurement An active measurement is indicated with a green diamond (◆)
PASS. REF.	The LED technology of the device does not require a passive reference. If you wish to measure a reference dye anyway, place a checkmark in this column.

MEAS. REPEATS	Enter the number of repetitions of the fluorescence measurement Possible values: 1 to 16
COLOR COMPENSATION	Activate spectral compensation (→ see section "Spectral compensation" p.32)
SCAN REGION ACCORDING LAYOUT	Sample measurement according to the layout of the samples on the SAMPLESTab (→ see section "Editing the sample table" p. 34).
DEFINE SCAN REGION MANUALLY	Sample measurement according to manual settings (→ see section "Manually defining the scan region" p.31)

The information on position, excitation, channel and detection and the selection list of the available dyes cannot be modified in this table. Modifications of these general settings can only be made in the EDIT COLOR MODULES window (→ see section "Editing color modules" p. 92).

Set the following parameters for each channel you wish to make a measurement for:

1. Select the dye to be measured in the DYE column. Click in the cell and mark the dye in the list that opens up.

   Note:
   The number of measured dyes does not have an influence on the scan time.

2. Set the signal quality in the GAIN column.
   The standard setting is "3".

3. Activate the fluorescence measurement in the channel in the "Measurement" column by placing a green diamond (◆).
   Channels that are not marked with a diamond will not be measured.

4. If necessary, activate the reference dye measurement by placing a (✓) in the PASS. REF. column.

5. Enter the number of repetitions for the fluorescence measurements in the MEAS. REPEATS field.
   The standard setting is "3".

   Note:
   An increased number of repeat measurements reduces the measurement value distribution but also creates longer scanning times and thus longer protocolling times.

6. Select one of the options for the SCAN REGION (MANUALLYOR ACCORDING TO LAYOUT).

3.3.1 Manually defining the scan region

Project window / Settings / Scan

The scan region can be defined according to the plate layout in the sample table (see section "Editing the sample table" p. 34) or manually. The scan region for the thermocycler is always defined per column. It must always consist of connected columns.

1. For a manual sample selection, select the DEFINE SCAN REGION MANUALLY option on the SETTINGS / SCANtab.

2. A graphical representation of the sample block is opened.

3. Enter the first and last column of the region to be scanned into the FROM COLUMN andTO COLUMN fields.

Optionally, you can use the mouse to select the columns. To select an individual column click directly into that column. If you wish to select several columns, press and hold the left mouse button and move the cursor over the corresponding area.

Active columns are highlighted blue in the diagram.

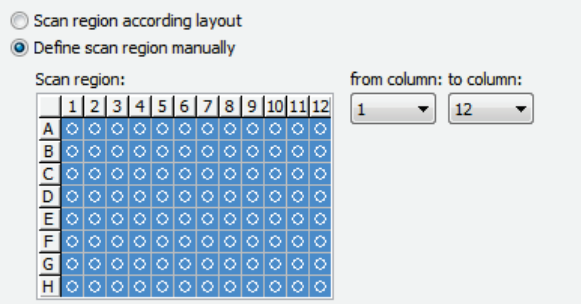


Fig. 21 Manual adjustment of the scan region

3.3.2 Color compensation

Project window / Settings / Scan

If you are using several dyes per reaction mixture the result may be a fluorescence crosstalk. This means that a second dye is excited and measured next to the desired dye at the same time. To subtract the fluorescence quotient of the second dye, you can use the color compensation function on THE SETTINGS / SCAN project tab. The standard setting for the color compensation is OFF. This is due to the fact that color compensation is not required for the most common applications (only one active measuring channel or dyes that are spectrally widely spaced, such as FAM and ROX). If you click on SELECT… a window opens up, in which color compensations that have already been recorded can be opened and used again.

In the window only those compensation data are printed in black color which meet the settings on the card scan. Invalid compensation data appear in red color and cannot be selected.

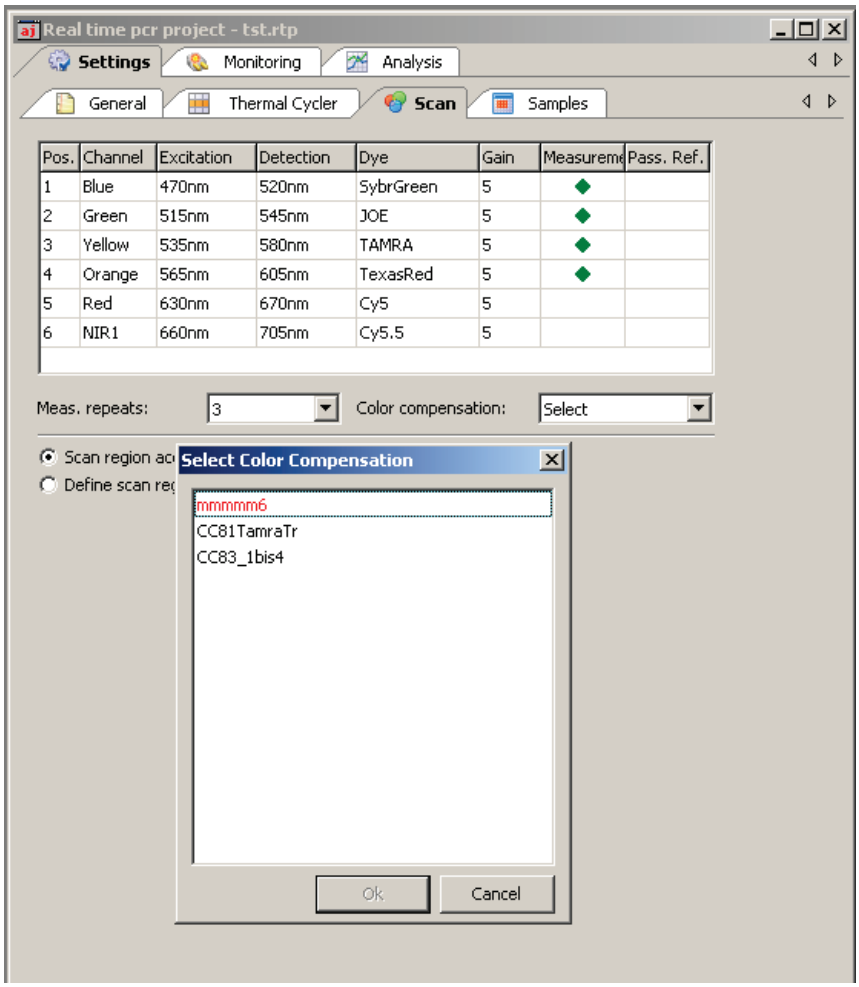


Fig. 22 Window for selection of color compensation data

Select a color compensation for the current project with the left mouse button and press [OK] to activate that compensation.

Scan / Edit color compensation

With the symbol or the menu command SCAN / EDIT COLOR COMPENSATION, you can create a new color compensation by measurement. This process is called spectral calibration. A new window opens, in which all the required settings can be made. The window is divided in a selection list for dyesand a plate diagram.

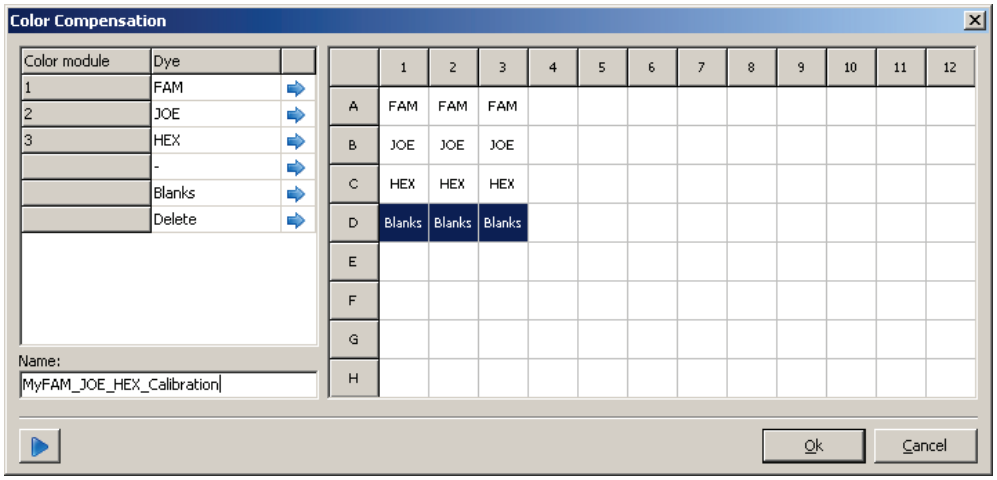


Fig.23 Color compensation for spectral calibration window

To record the calibration data the dyes required for a color compensation must be available individually in solution. You could for example use the sensors for the

calibration measurement that are to be used in the later PCR experiment. The dye concentration for the calibration measurement should be approx. 0.1 µmol/l.

In the displayed plate diagram the wells that contain the calibration samples are now marked individually for each dye and the dye in each of the samples is assigned to the marked well by clicking on the blue arrow. The dyes offered for selection are those that were selected in the SETTINGS / SCAN project tab.

For an exact calibration measurement we recommend that each dye be created at least as a triple replicate as well as at least three blanks (sample containers filled with water).

The calibration measurement is started by clicking on the blue triangle on the bottom left .

Note:
The selection of available dyes cannot be modified in this table. Modifications can only be made on SCAN tab.

The new color compensation needs to be assigned a description in the name field. It is included in the selection list after pressing [OK] and will be displayed in the corresponding window. Templates that are no longer in use can be deleted with [DELETE].

3.4 Editing the sample table

Project window / Settings / Samples

The sample table defines which sample is in which positionof the block. These details are required for using the evaluation functions of qPCRsoft. Here, a sample can be described by means of its properties, such as name, gene, type, concentration and dye. Furthermore, samples from different experimental approaches can be combined in groups.

The necessary entries can be made on the SAMPLES tab after pressing the [EDIT LAYOUT] button. The corresponding window is divided into different sections:

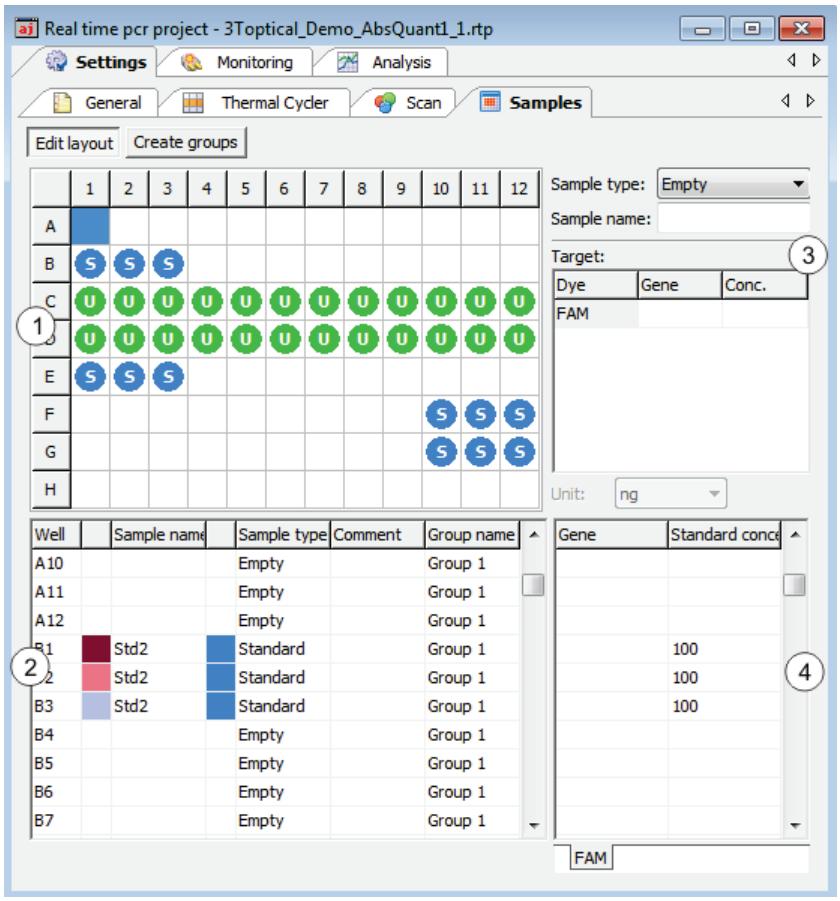


Fig. 24 Window for editing the sample table

Range	Function
LAYOUT VIEW (1)	Graphical display of the well assignment on the microplate
EDIT AREA (3)	Edit area for the sample properties: • Sample name • Sample type • Concentration of standard samples • Allocation of the dye and the analyzed gene
SAMPLE TABLE (2)	Summary of the information on each sample
DYES (4)	Dyes and assigned genes for each sample

Note:
The sample table can also be edited after the Real-Time PCR run has been completed.

3.4.1 Defining sample properties

Entering sample properties in the layout

Settings / Samples project tab

You can define the properties for the samples in the wells on the SETTINGS / SAMPLES project tab in the layout view and the edit area next to it.

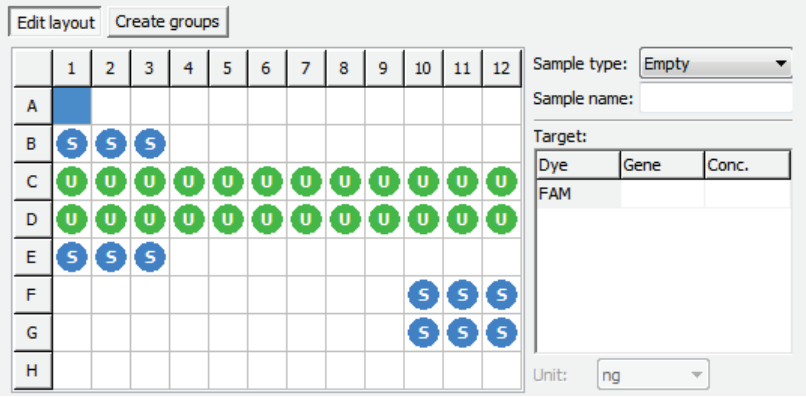


Fig.25 Layout view for the sample type

The following sample types can be defined:

Sample type	Symbol	Definition
EMPTY		Describes an empty position on the PCR plate
UNKNOWN	U	Sample of unknown concentration or dilution (measuring sample)
STANDARD	S	Sample of known concentration or dilution
CALIBRATOR	K	Sample the target gene expression level of which is set to 1
NO TEMPLATE CONTROL (NTC)	N	Complete reaction mixture but without template strand
POSITIVE CONTROL	+	Positive control assay for which a reaction product can be expected
NEGATIVE CONTROL	-	Negative control assay for which no reaction product is to be expected

Samples with identical sample properties (sample name, sample type, same gene-dye-assignments) are viewed as multiple measurements. The individual values of these samples are averaged and their mean value is used for the remaining calculations.

With a singleplex assay, samples can have the same sample name and sample type but differ as far as the gene-dye-assignment is concerned. These samples are identified as associated samples due to the same name. The evaluation, however, is performed individually.

❑ Mark the sample position to be edited by clicking into the layout.
Multiple selections are only possible in adjoining fields. To do so, press and hold the left mouse button and move the cursor over the corresponding fields.

To mark rows or columns, click on the corresponding row or column name [A-H] or [1-12].

You can mark all sample positions in the layout by clicking on the gray button in the top left of the layout (between 1 and A1).

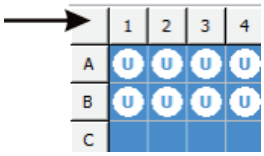


Fig. 26 Button for marking the complete layout

❑ Enter the following sample parameters in the adjacent edit area:

Parameter	Description
SAMPLE NAME	Sample description
SAMPLE TYPE	Selection of the sample type (see top of table)
TARGET table	
GENE column	In the dye row, enter the gene to be analyzed
CONC. column	*For standards* Enter the concentration of the gene to be analyzed.

❑ You can assign sample properties to the marked positions by clicking on the symbol in the toolbar.

Note:
The entries for the selected area will only be applied by the program after they have been assigned. Entries or modifications that are not assigned will be lost.

❑ To display defined sample properties in the edit area, double-click on a well.

You can edit the information and assign them to the well again by clicking .

To assign these sample properties to other wells, mark the desired wells and then click .

The entry of a sample layout is described by means of an example for a singleplex assay (→ "Entering a sample layout for a singleplex assay" S. 40 and a multiplex assay "Entering a sample layout for a multiplex assay" S. 38).

Entering sample properties into the sample table

You can also make entries in the sample table itself. Select the desired position in the layout view or a field directly in the sample table. The corresponding row is then highlighted yellow. Descriptions and/or values can be defined directly in the designated cells. The sample table is edited cell by cell. Multiple selections and the associated assignment of parameters to several cells or rows at a time are not possible.

Well		Probenname		Probentyp	Bemerkung	Gruppennam
A1		Zellkultur 24h		Standard		Group 1
A2		Zellkultur 24h		Standard		Group 1
A3		Zellkultur 24h		Standard		Group 1
A4		Zellkultur 24h		Standard		Group 1
A5		Zellkultur 24h		Standard		Group 1
A6				Unbekannt		Group 1
A7				Unbekannt		Group 1
A8				Unbekannt		Group 1

Gen	Standardkonze
GAPDH	100
GAPDH	100
GAPDH	100
GAPDH	100
GAPDH	100

FAM JOE HEX ROX Cy

Fig.27 Sample table for entering sample properties

The genes and, in the case of standard samples, their concentration are summarized separately by dye in the second part of the sample table. A list sheet is assigned to each dye. It is thus possible to use different standard concentrations for each gene. The number of displayed sheets depends on which dyes have been activated on the SETTINGS / SCAN project tab for this measurement.

Depending on the selected window size and the number of dyes to be measured, two buttons with left and right arrows appear. They can be used to move between the different tabs.

The number of columns that are displayed in the sample table can be user-defined. Right-click on a column header to display the following selection field:

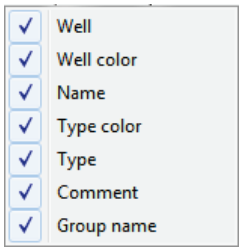


Fig.28 Selection field for defining the columns displayed in the sample table.

You can select or deselect individual columns to show or hide a column and modify the representation as desired.The order of the columns can be changed by clicking on the table header and dragging with the mouse.

Displaying sample properties in the project explorer

Moving the mouse pointer over a well on the SAMPLE project tab in the layout view will display the properties of the well in the SAMPLES menu item in the project explorer.

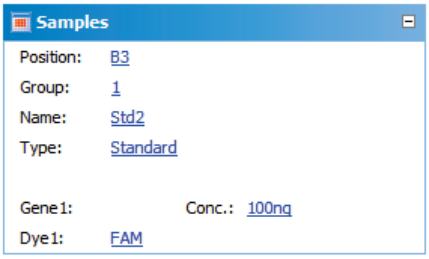


Fig. 29 Project explorer Samples window showing the well properties

Entering a sample layout for a multiplex assay

The following example shows the definition of four samples and standards with three repeat measurements each in the layout. The GAPDH gene is analyzed with the FAM dye and the c-myc gene with the VIC dye. The two dyes are selected on the SETTINGS / SCAN project tab and activated for measurement. The indicated sample names and standard concentrations serve as examples only.

1. Activate the [EDIT LAYOUT] button.
2. Empty the plate layout to make sure that no unintentional entries remain:
   - Mark the complete plate by clicking on the gray button in the top left of the layout.
   - In the SAMPLE TYPE list select the option EMPTY.
   - Click on .

3. Mark the three wells A1 - A3.
4. Make the following settings:

Parameter	Entered value
SAMPLE NAME	Sample 1
SAMPLE TYPE	Unknown
FAM	GAPDH
VIC	c-myc

Note:
The genes are allocated to the corresponding dye by entering the name of the gene or by selecting it from the displayed list in the TARGET table.

5. Click on , to assign the sample properties to the three wells.
6. Repeat steps (3) - (5) for the other samples.
Use the following parameters:

Wells	Sample name	Sample type	FAM	VIC
A4 – A6	Sample 2	Unknown	GAPDH	c-myc
B1-B3	Sample 3	Unknown	GAPDH	c-myc
B4-B6	Sample 4	Unknown	GAPDH	c-myc

Defining the standard samples

7. Mark the three wells C1 - C3.
8. Make the following settings:

Parameter	Entered value
Sample name	Std 1
Sample type	Standard

Make the following entries in the TARGET table:

Dye	Gene	Conc.
FAM	GAPDH	100
VIC	c-myc	50

Note:
The CONC. column in the TARGET table is only available for the STANDARD sample type.

9. From the UNIT list, choose a concentration or mass unit. You can select from the following units:ng, ng/µl, ng/ml, pg/µl, copies, copies/µl, copies/ml, mg/ml, IU/µl, IU/ml or %.
10. Click on , to assign the sample properties to the three wells.
11. Repeat steps (7) - (10) for the other three standards.
Use the following parameters:

Wells	Sample name	Conc.	
		FAM	VIC
C4 – C6	Std 2	50	5
D1 - D3	Std 3	10	1
D4 - D6	Std 4	0.1	0.05

As sample type, select the STANDARD option.

Assign the genes to the dyes as described in step (6).

✓ **The plate layout for a multiplex assay is complete.**

Entering a sample layout for a singleplex assay

The following example shows the definition of four samples and four standards with three repeat measurements each in the layout. The GAPDH and c-myc genes are analyzed with the FAM dye with the help of two sensors. The FAM dye was selected on the SETTINGS / SCAN project tab and activated for the measurement. The indicated sample names and standard concentrations serve as examples only.

1. Activate the [EDIT LAYOUT] button.
2. Empty the plate layout to make sure that no unintentional entries remain:
   - Mark the complete plate by clicking on the gray button in the top left of the layout.
   - In the SAMPLE TYPE list select the option EMPTY.
   - Click on .
3. Mark the three wells A1 - A3.
4. Make the following settings:

Parameter	Entered value
Sample name	Sample 1
Sample type	Unknown
FAM	GAPDH

5. Click on , to assign the sample properties to the three wells.
6. Mark the three wells A4 - A6.
7. Make the following settings:

Parameter	Entered value
Sample name	Sample 1
Sample type	Unknown
FAM	c-myc

8. Click on , to assign the sample properties to the three wells.
9. Repeat steps (3) - (8) for the other three samples.
   Use the following parameters:

Wells	Sample name	Sample type	Gene /FAM
B1-B3	Sample 2	Unknown	GAPDH
B4-B6	Sample 2	Unknown	c-myc
C1-C3	Sample 3	Unknown	GAPDH
C4-C6	Sample 3	Unknown	c-myc
D1-D3	Sample 4	Unknown	GAPDH

D4-D6	Sample 4	Unknown	c-myc

Defining the standard samples

10. Mark the three wells E1 - E3.
11. Make the following settings:

Parameter	Entered value
Sample name	Std1
Sample type	Standard

Make the following entries in the **Target** table:

Dye	Gene	Conc.
FAM	GAPDH	100

Note:
The CONC**.** column in the TARGET table is only available for the STANDARD sample type.

12. From the UNIT list, choose a concentration or mass unit. You can select from the following units:ng, ng/µl, ng/ml, pg/µl, copies, copies/µl, copies/ml, mg/ml, IU/µl, IU/ml or %.
13. Click on , to assign the sample properties to the three wells.
14. Repeat steps (10) - (13) for the other standards.
Use the following parameters:

Wells	Sample name	Gene	Conc.
E4 – E6	Std. 1	c-myc	100
F1 – F3	Std. 2	GAPDH	75
F4 – F6	Std. 2	c-myc	75
G1 – G3	Std. 3	GAPDH	50
G4 – G6	Std. 3	c-myc	50
H1 – H3	Std. 4	GAPDH	10
H4 – H6	Std. 4	c-myc	10

15. Click on , to assign the sample properties to the three values.

Note: Connected samples must have the same sample name.

✓ **The plate layout for a singleplex assay is complete.**

3.4.2 Defining groups

Several experiments can be performed on one microplate at the same time. The samples that are part of one experiment are combined in a group. A group contains a number of reaction mixtures that will be evaluated together later on. You can define a maximum of 12 such groups.

The groups are defined in the SETTINGS / SAMPLES project window.

1. In the SETTINGS / SAMPLES project window click on the [CREATE GROUPS] button.

The GROUP list and the GROUP NAME field are activated.
In the layout, all samples are marked with the number 1. This means they have been assigned to group 1.

2. In the layout, mark the samples that are part of one experiment.
   To mark adjacent samples, press and hold the mouse button.
3. Select the next group from the GROUP list.
4. Enter the description for the experiment in the GROUP NAME field. You may select any group name.
5. Click on , to assign the group properties to the samples.

   The samples that belong together are marked with the group number in the layout.

   The descriptions are displayed in the sample table in the GROUP NAME column.

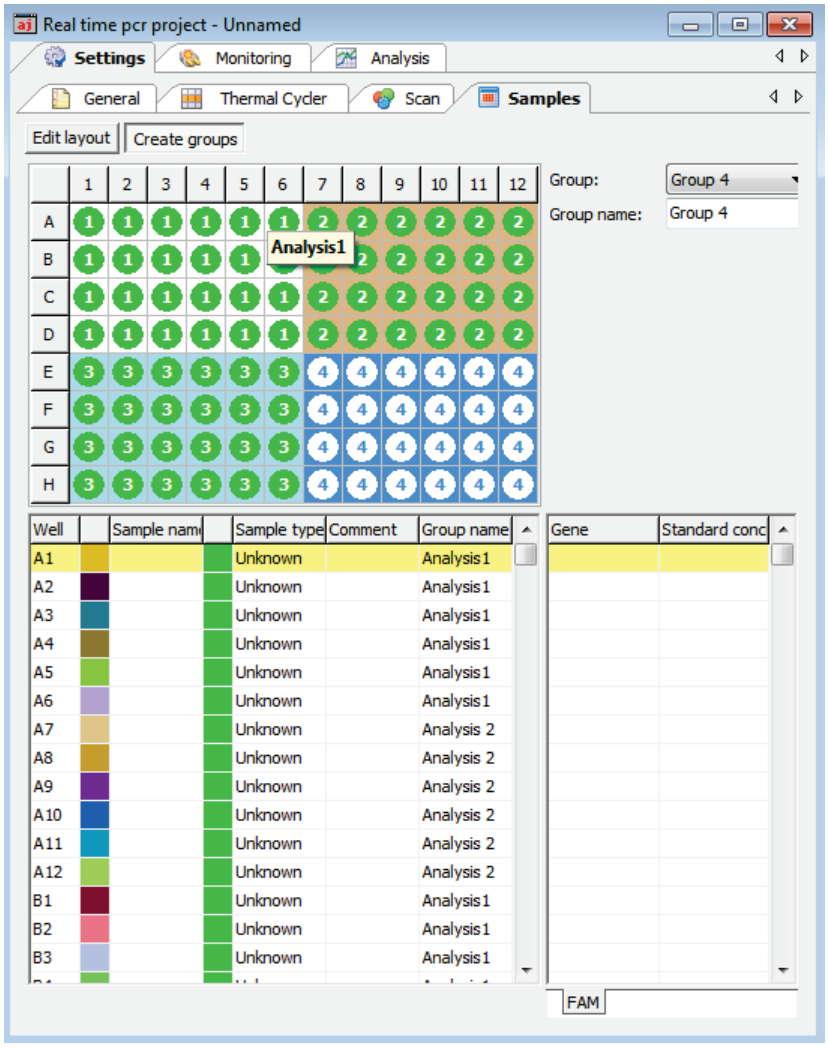


Fig.30 Sample layout with four groups

3.4.3 Layout preview

The layout preview provides a complete overview of the layout of the PCR plate with samples and the corresponding information that has been saved for the samples.

Samples / Preview layout

❑ Open the layout preview by clicking on the symbol in the toolbar.
Optionally, call up the SAMPLES / PREVIEW LAYOUT menu command.

The layout preview is displayed in the VIEW SAMPLE PLATE window.

The layout preview provides an overview of the following properties:

- Position on the PCR plate
- Genes to be determined
- Sample type by means of color marking at the edge
- Underlined in color to indicate group affiliation

If you move the cursor to a specific position, all known settings for this position, such as sample names, sample type and group and all genes and dyes to be measured for the sample as well as the concentration in the case of standards are displayed in detail.

	1	2	3	4	5
A	S Zellkultur 24h FAM: GAPDH	S Zellkultur 24h FAM: GAPDH	Zellkultur 24h Position: A2	Zellkultur 24h	Zellkultur 24h FAM: GAPDH
B	S Zellkultur 24h FAM: GAPDH	S Zellkultur 24h FAM: GAPDH	Gruppe: 1 Name: Zellkultur 24h		Zellkultur 24h FAM: GAPDH
C	U Zellkultur 24h FAM: MCF-1	U Zellkultur 24h FAM: MCF-1	Typ: Standard		Zellkultur 24h FAM: MCF-1
D	U Zellkultur 24h FAM: MCF-1	U Zellkultur 24h FAM: MCF-1	Gen1: GAPDH Konz.: 100ng Farbstoff1: FAM		Zellkultur 24h FAM: MCF-1

Fig.31 Section from the layout preview table

The table can be printed and used, for example, as a template for pipetting the samples or for documenting the experiment.

❑ Press the [PRINT] button in the VIEW SAMPLE PLATE window to print the table.

3.4.4 Copying the layout

The layout view or parts of the layout can be copied and inserted at different positions in the same layout or into the layout of another project.

1. Use your mouse to mark the area in the layout view to be copied.

Samples / Copy layout

2. Transfer the information to the clipboard by clicking on the symbol in the toolbar.
Optionally, call up the SAMPLES / COPY LAYOUT menu command.

3. Select a position for inserting the copied area. The selected position or the left upper position of the selected area determines the position at which the copied section in the layout view is to be inserted.

Samples / Paste layout

4. Paste the information using the symbol.
Optionally, you can use the SAMPLES / PASTE LAYOUT menu command.

The way described to edit the layout is related to the graphical presentation of the PCR plate in the upper part of the projrct window. If the layout table shall be edited by copy, cut and paste this can be done by using the right hand mouse button and keeping the Ctr-button pressed.

5. Press the Ctr-button and hold it pressed during the whole operation.
6. Mark the lines to be copied or cut by left-klicking the mouse and drawing it up or down.
7. Press the right mouse button and select the desired function

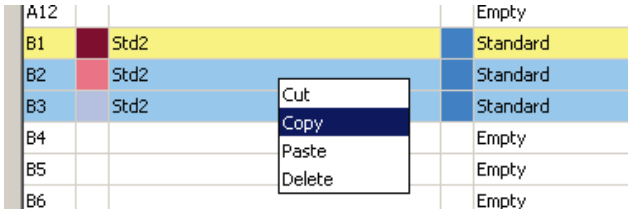


Fig. 32 The standard samples in the wells B1, B2 and B3 are copied to the clipboard

8. Select the line by left clicking the mouse from which the copied samples shall be inserted. By right clicking the mouse the paste menu appears.

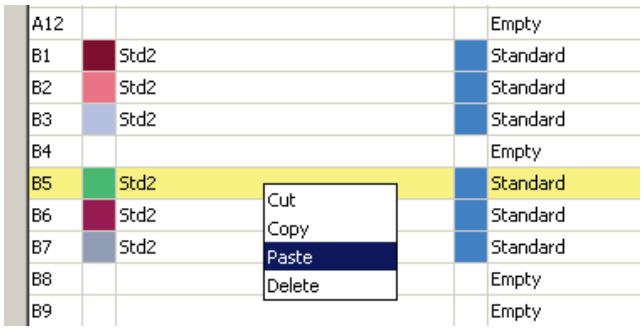


Fig. 33 The selected standard samples including their properties are now copied to the wells B5, B6 and B7. Please note that caused by their identical sample name the standard Std2 is now a six fold replicate.

3.4.5 Exporting or importing the layout in Excel

The layout can be exported or imported as an Excel file (*.xls). The exported data can be edited in Excel and then reimported.

❑ Right-click on the sample table.

A context menu with the IMPORT TABLE FROM EXCEL FILE (*.XLS) and EXPORT TABLE TO EXCEL FILE (*.XLS) menu commands opens.

Select the desired menu command.

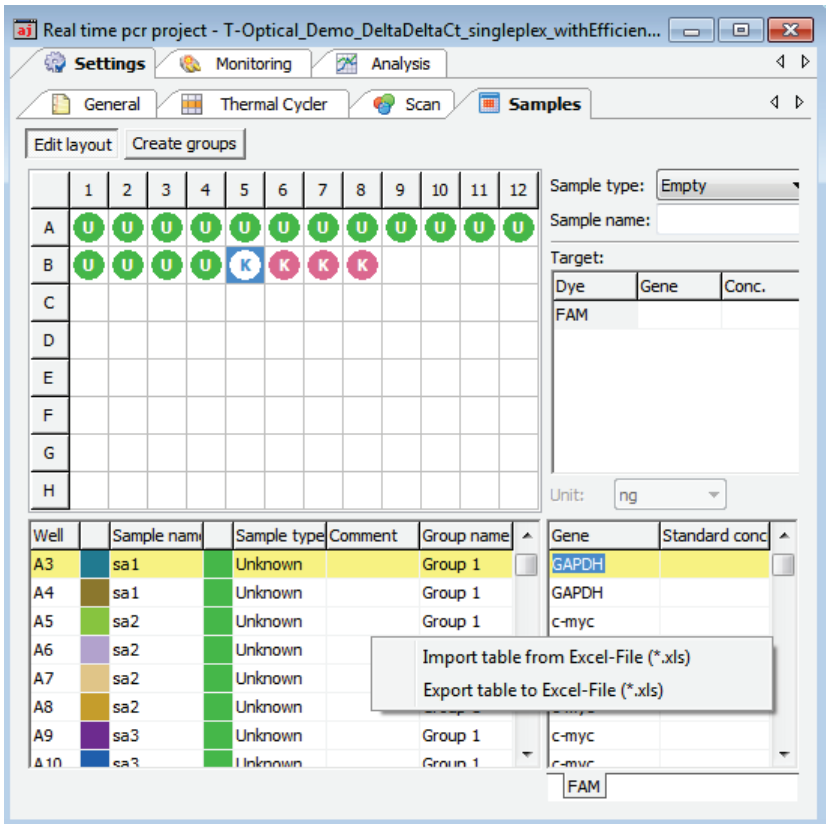


Fig. 34 Excel export and import function for the sample layout

4 Monitoring

All functions required for starting and monitoring a Real-Time PCR run are combined on the MONITORING project tab.

4.1 Starting the PCR protocol

Activate the MONITORING tab in the project window to display the symbols for starting the PCR and the MONITORING menu protocol defined in the SETTINGS project window in the tool bar.

Symbol	MONITORING / … menu command	Description
	START QPCR RUN	Start the PCR run
	STOP QPCR RUN	The PCR run is interrupted and will not be continued. The data recorded up to this point is saved and can be evaluated.
	PAUSE QPCR RUN	The PCR run is interrupted. The symbol flashes during the break. The PCR run can be continued by clicking again.
		QTower only Opens and closes the thermoblock.
	VIEW OPTIONS	Defines default settings for the Monitoring view.

Warning! Contamination of the thermoblock!

Do not leave the thermoblock open unless absolutely necessary. There is a risk that the thermoblock becomes contaminated. This will lead to a poorer quality and efficiency of the optical fluorescence measurement.

4.2 Display options for monitoring

Monitoring project window

The MONITORING project window is divided into the following areas:

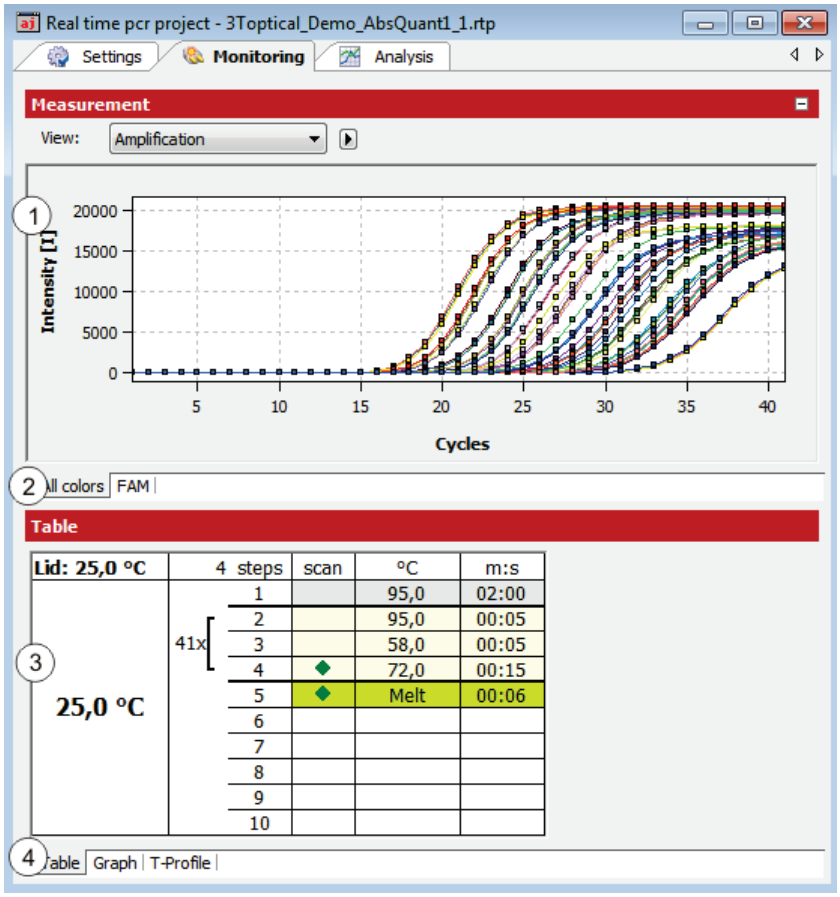


Fig.35 Monitoring project window for monitoring PCR runs

Area	Function
MEASURING RESULTS (1)	Displays the measured fluorescence data. The fluorescence intensity is plotted against the cycle.
COLORS tabs (2)	Switches between the fluorescence accumulation curves that were measured for the individual dyes.
PCR PROTOCOL (3)	Displays the PCR protocol. The active step is indicated by a green arrow.
PROTOCOL VIEW tabs (4)	Switches between different views of the PCR protocol (tabular, graphic, temperature profile).

The fluorescence measurements are displayed in the top area. On the different list sheets you can choose between the overlapping display of the measurement results of all dyes or the display of the individual dyes. In the VIEW list you can switch between the AMPLIFICATION, MELTING CURVE and RAW DATA views.

The current PCR protocol is displayed at the bottom of the table in the project window. The active step is marked by means of a green arrow during the PCR run. On the different sheets you can choose between a tabular or graphical view of the PCR protocol or the temperature profile. The view of the PCR protocol is described in the section "Monitoring the PCR run" on page 51.

4.2.1 Default settings for the Monitoring view

For all views in the MONITORING project window it is generally possible to choose between linear and logarithmic scaling for the graphical display of the data. The setting for the baseline correction can also be changed.

Monitoring / Display options

❑ Select the MONITORING / DISPLAY OPTIONS menu command or click in the toolbar.

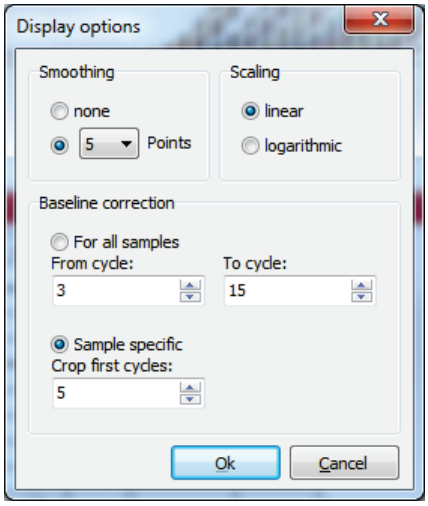


Fig. 36 DISPLAY OPTIONS window for changing the default settings of the Monitoring view

Parameter / Option	Description
SMOOTHING	Setting the smoothing conditions for the measured data
SCALING	Scaling options for the data (LINEAR or LOGARITHMIC)
BASELINE CORRECTION	There are two options for the baseline correction: – FOR ALL SAMPLES If this option is selected, the baseline for every sample in the same range is determined. The upper and lower range limit must be set in the FROM CYCLE and TO CYCLE fields. – SAMPLE SPECIFIC Select this option if the curves have significantly different Ct values. The lower range limit for determining the baseline is set in the CROP FIRST CYCLES field for all samples. The upper range limit is determined separately for each sample by an algorithm. **Note:** The type of baseline correction can only be set in this dialog. The range limits for the correction can also be adjusted in the project window.

4.2.2 Adjusting the view in the MONITORING project window

In the Monitoring project window, you can adjust the preset parameters (MONITORING / DISPLAY OPTIONS menu command) for the display of the scaling as well as the range limits for the baseline correction.

1. Click on the button above the chart.

   A selection field for setting the display options and entering the baseline parameters opens.

FOR ALL SAMPLES baseline correction parameter

SAMPLE SPECIFIC baseline correction parameter

Fig. 37 Parameter settings in the MONITORING project window

2. Change the baseline correction limits and activate the LINEAR or LOGARITHMIC option for the desired view of the fluorescence curves.

4.2.3 Displaying and hiding measurement results for individual wells

The sample view in the MONITORING project window is controlled via the SAMPLES menu item in the project explorer. Measurement results in the individual wells can be hidden or shown.

Note:
The selection in the project explorer only influences the display of the fluorescence data but not the measurement.

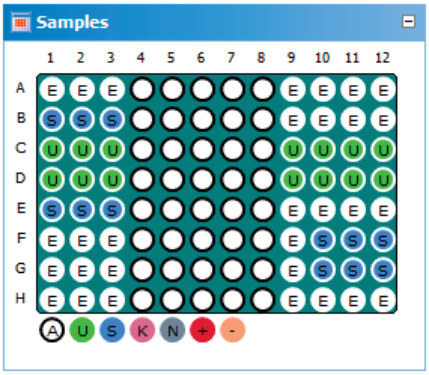


Fig. 38 Project explorer SAMPLES window

The marking of the sample assignment corresponds to the marking on the SAMPLES project tab:

Sample type	Symbol	Definition
EMPTY	E	Describes an empty position on the PCR plate
UNKNOWN	U	Sample of unknown concentration or dilution (measuring sample)
STANDARD	S	Sample of known concentration or dilution
CALIBRATOR	K	Sample whose gene of interest expression level is set as 1
NO TEMPLATE CONTROL (NTC)	N	Complete reaction preparation but without matrix strand
POSITIVE CONTROL	+	Positive control preparation for which a reaction product is expected

NEGATIVE CONTROL	-	Negative control preparation for which no reaction product is expected

Active wells (i.e. displayed wells) are marked with their sample type symbol. For deactivated wells the position is white and the fluorescence data is hidden. Empty wells are marked "E". No data is saved for empty wells.

- Click with the mouse to switch. The activation changes with each click on a well.
- You can change adjacent wells by holding the mouse button and moving the cursor over the wells.
- Complete rows and columns can be inverted by clicking on the letter or number of the row [A-H] or column [1-12].
- The complete plate can be switched by clicking into the empty field on the top left between A and 1.
- To activate all wells click on the symbol below the chart.
- To activate only samples of a specific type click on the corresponding symbol below the chart. To activate multiple sample types at the same time keep the Ctrl key pressed when clicking on the sample types.

4.2.4 Exporting fluorescence data

The data from the fluorescence measurement can be exported as ".csv" files. In addition, the graphical display of the measurement results can be copied to the clipboard as a hardcopy and is hereby made available for other programs.

- Right-click on the graph.
- A selection window for export and hardcopy appears.
- Click on COPY CHART to copy the chart to the clipboard.
- Select the SAVE CHART option to export the fluorescence data.
  The SAVE AS standard window opens.
  Enter a file name and confirm with [OK].

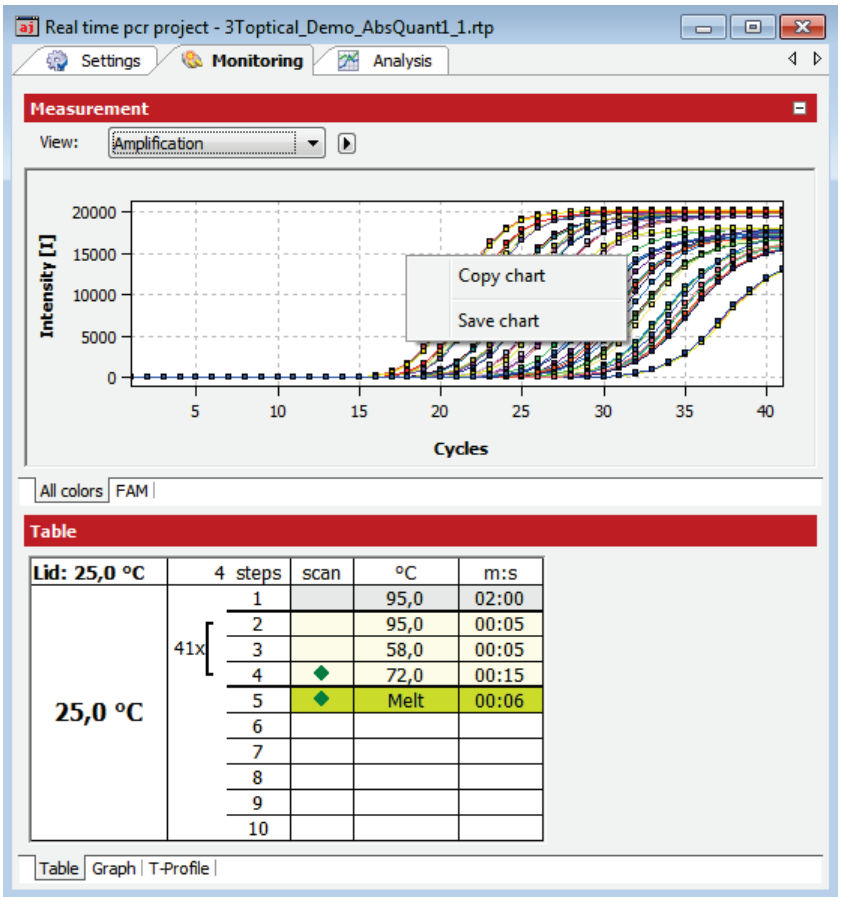


Fig. 39 Context menu for exporting the fluorescence data in the MONITORING project window

4.3 Monitoring the PCR run

The running PCR protocol is shown in the bottom part of the MONITORING project tab. It is generally possible to to choose between the three different views (list sheets) GRAPH, TABLE and T-PROFILE via tabs.

In addition to the display on the list sheets a status bar shows further information on the protocol, such as the plateau time, the calculated remaining time and, in programmed loops, the step number and the number of loops.

Fig. 40 Status bar of the MONITORING project tab

TABLE list sheet

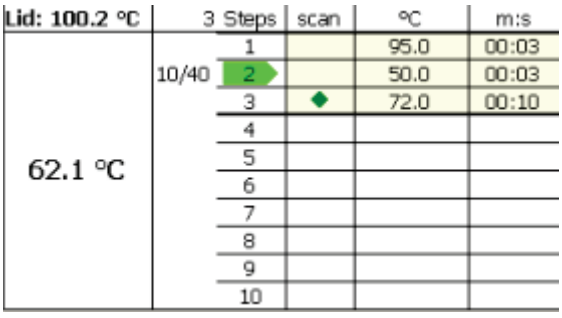


Fig. 41 Monitoring project window with PCR protocol table

Element	Description
LID	Current lid temperature
TEMPERATURE DISPLAY	Current block temperature
STEPS	Temperature steps in the PCR protocol The active step is marked by a green arrow.
°C	Target temperature of the step
M:S	Hold time, format: min:s

GRAPH list sheet

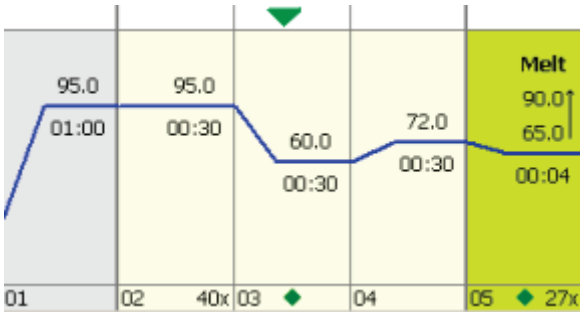


Fig. 42 Monitoring project window with PCR protocol table

The GRAPH list sheet contains the same elements of the graphical representation of the PCR protocol as the SETTINGS / THERMOCYCLER / GRAPH project window. Once again, the active step is marked by a green arrow.

T PROFILE list sheet

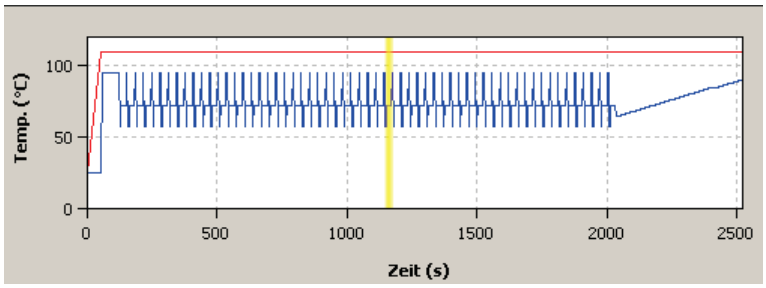


Fig. 43 Monitoring project window with T profile

In the representation of the temperature profile a yellow progress bar indicates the step that is currently being performed.

4.4 Displaying product accumulation curves

The product accumulation is documented by means of fluorescence measurements during the PCR run.

To display measurement curves for the product accumulation select the AMPLIFICATION or RAW DATA option from the VIEW list. In the chart the fluorescence intensity [I] is plotted against the number of cycles in relative units. The color of the curve that is being displayed corresponds to the color assigned to each well in the sample table (SETTINGS / SAMPLE project window).

The measurement results for the individual dyes are selected via the corresponding list sheets. You have the option to display the measurement results of all dyes together (ALL COLORS list sheet) or only the results of an individual dye.

The display options for the product accumulation curves are described in the "Display options for monitoring" section on page 47.

4.5 Displaying melting curves

To display the melting curves, select the MELTING CURVE option from the VIEW list.

The display options for the product accumulation curves are described in the "Display options for monitoring" section on page 47.

5 Evaluation

On the ANALYSIS tab of the project window the following methods are available for evaluating Real-Time PCR experiments on four cards:

- Absolute quantification
- Relative quantification
- ΔΔCt method
- Melting curve determination
- Genotyping

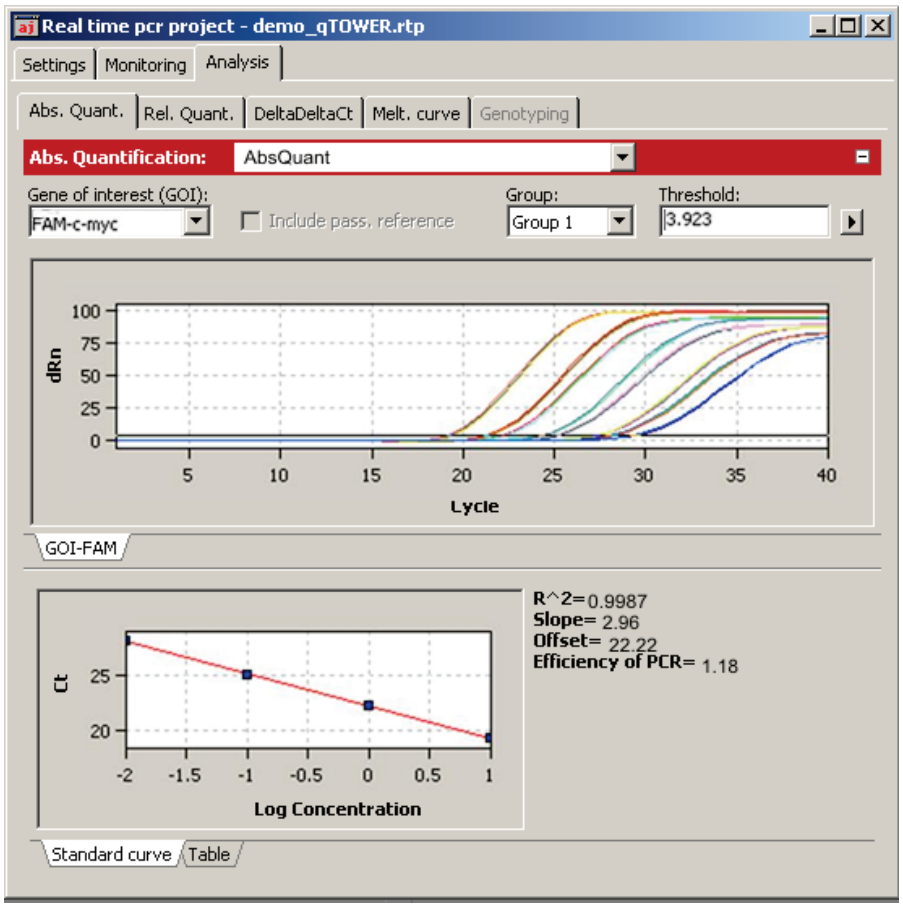


Fig.44 Evaluation project window

The individual evaluation methods can be accessed via the subordinated tabs. For each selection method different evaluations can be created.

Toolbar and menu commands are adjusted to the requirements of the selected method tab.

5.1 General functions in the evaluation project window

5.1.1 Making basic settings

Presets can be made for some evaluation parameters.

1. Click on in the toolbar.
2. Set up the following parameters.

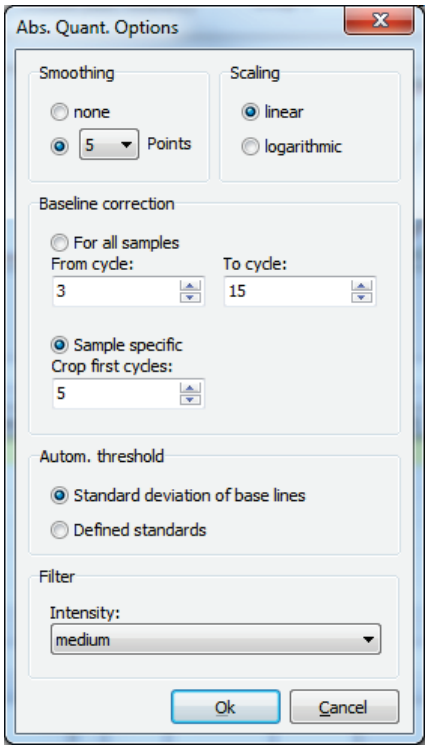
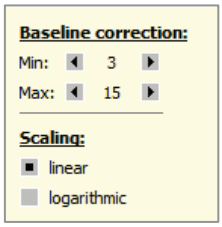


Fig. 45 Window for basic evaluation settings

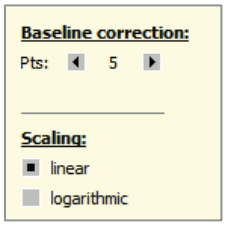
Option	Function
SMOOTHING	Smoothing the fluorescence curves on the basis of the calculated moving average over a range of 2 to 12 measuring points or representation without smoothing
SCALING	Linear or logarithmic representation of fluorescence curves
BASELINE CORRECTION	Bei der Korrektur der Basislinie ist zwischen zwei Optionen für die Art der Korrektur zu wählen At the correction of the base line you can choose between two options: – FOR ALL SAMPLES Bei dieser Korrektur wird die Basislinie für jede Probe im gleichen Bereich ermittelt. Die untere und obere Bereichsgrenze sind in den Feldern VON ZYKLUS und BIS ZYKLUS einzustellen. At this correction the base line is determined for every sample in the same area. The lower and upper area limit has to be edited in the fields FROM CYCLE and TO CYCLE. – SAMPLE SPECIFIC This correction should be chosen if the curves have very different Ct values. The lower area limit for the determination of the base line will adjusted in the field CROP FIRST CYCLES for all samples. The upper area limit is separately found out by an algorithm for each sample. **Note:** The manner of the base line correction can be selected only in this dialog. The area limits can, however, be adapted for the correction in the project window.
AUTOM. THRESHOLD	Calculation of the threshold as a deviation of x times of the standard deviation of the baselines (factor can be adjusted under EXTRAS / OPTIONS / ANALYSIS in the main menu) or based on defined standards with the goal to get the maximum value for the coefficient of determination R^2

FILTER	Digital filter for smoothing the fluorescence curves. Adjustable in steps: SLIGHT, MEDIUM and STRONG

More setting options may be available depending on the analysis method used. They are explained separately in the respective sections. All items displayed on the ANALYSIS project tab can also be accessed quickly via the settings area of the baseline and displayed as linear or logarithmic representations. For this purpose, a selection for display options can be opened in the corresponding window via the right-arrow button ▶:



FOR ALL SAMPLES baseline correction parameter



SAMPLE SPECIFIC baseline correction parameter

Fig. 46 Individual setting of the baseline correction and fluorescence curve scaling during the evaluation

5.1.2 Activating/deactivating samples for evaluation

Samples from individual wells can be activated or deactivated for evaluation in the project explorer SAMPLES menu item. This enables you, for example, not to include excessive values when calculating mean values.

Note:
The selection in the project explorer only influences the analysis of the fluorescence data. Measured data will not be deleted.

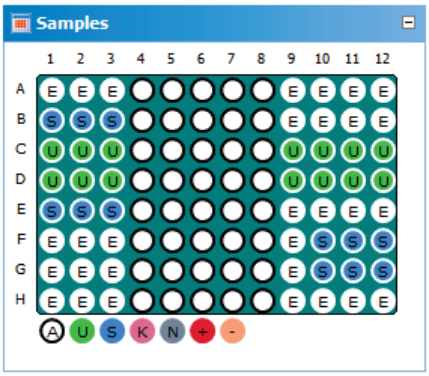


Fig. 47 Project explorer SAMPLES window for (de-)activating the samples in the evaluation

The marking of the sample assignment corresponds to the marking on the SAMPLES project tab:

Sample type	Symbol	Definition
EMPTY	E	Describes an empty position on the PCR plate
UNKNOWN	U	Sample of unknown concentration or dilution (measuring sample)
STANDARD	S	Sample of known concentration or dilution
CALIBRATOR	K	Sample whose gene of interest expression level is set as 1
NO TEMPLATE CONTROL (NTC)	N	Complete reaction preparation but without matrix strand

POSITIVE CONTROL	+	Positive control preparation for which a reaction product is expected
NEGATIVE CONTROL	-	Negative control preparation for which no reaction product is expected

Active wells (i.e. wells included in the analysis) are marked with their sample type symbol. For deactivated wells the position is white and the fluorescence data is hidden. Empty wells are marked "E".

- ❑ Click with the mouse to switch. The activation changes with each click on a well.
- ❑ You can change adjacent wells by holding the mouse button and moving the cursor over the wells.
- ❑ Complete rows and columns can be inverted by clicking on the letter or number of the row [A-H] or column [1-12].
- ❑ The complete plate can be switched by clicking into the empty field on the top left between A and 1.
- ❑ To activate all wells click on the Ⓐ symbol below the chart.
- ❑ To activate only samples of a specific type click on the corresponding symbol below the chart. To activate multiple sample types at the same time keep the Ctrl key pressed when clicking on the sample types.

5.1.3 Exporting evaluation data

The data from the fluorescence measurement can be exported as ".csv" files. In addition, the graphical display of the measurement results can be copied to the clipboard as a hardcopy and is hereby made available for other programs.

- ❑ Right-click on the graph.
- ❑ A selection window for export and hardcopy appears.
- ❑ Click on COPY CHART to copy the chart to the clipboard.
- ❑ Select the SAVE CHART option to export the fluorescence data.
  The SAVE AS standard window opens.
  Enter a file name and confirm with [OK].

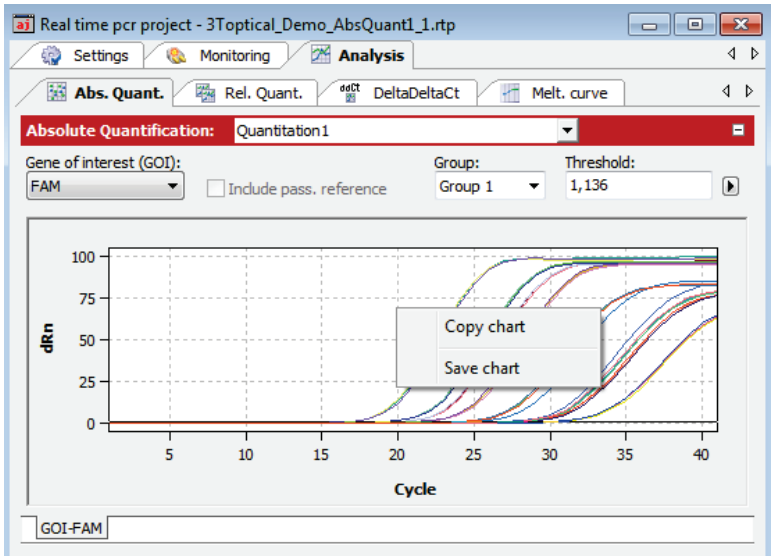


Fig. 48 Exporting data from the evaluation

5.2 Absolute quantification

The absolute quantification is used to determine absolute copy numbers in samples with the help of the comparison with standards with known concentrations.

5.2.1 Creating an evaluation for an absolute quantification

AbsQuant / Add abs. quantification

1. Go to the ANALYSIS / ABS QUANT project tab.
   If the tab is not visible, click on the arrows in the tab bar. This will scroll the tabs.
2. Click on the symbol in the toolbar.
   Optionally, call up the ABSQUANT / ADD ABS. QUANTIFICATION menu command.
3. An input window appears. Enter the description for the current evaluation.

   On the ABSQUANT tab the following information is activated:

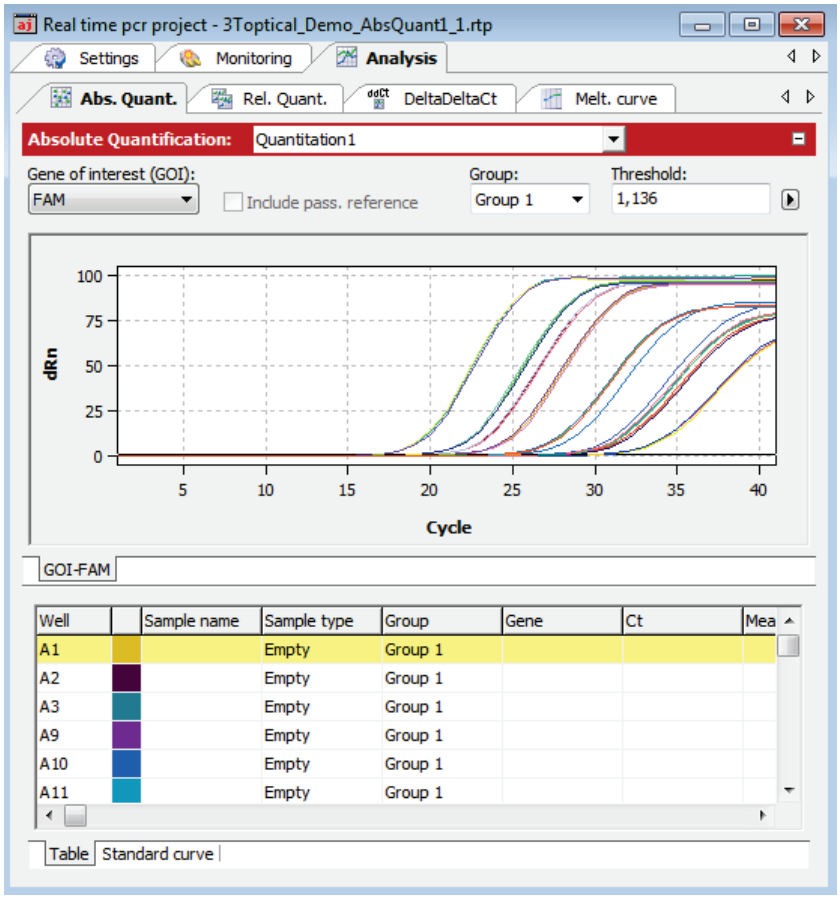


Fig.49 Window for absolute quantification

- Parameter settings
- Display of fluorescence spectra
- Display of sample table with analysis results
- Display of the regression lines and the calculated coefficients

5.2.2 Parameter settings for absolute quantification

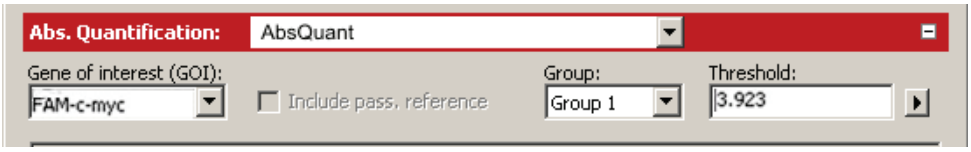


Fig.50 Parameter settings for absolute quantification

Set the following parameters for the absolute quantification:

Option	Description
SELECTION LIST	Selection of an evaluation created for the experiment
GENE OF INTEREST (GOI)	Selection list of target gene/dye combinations According to the selection, the fluorescence and regression curves for the concentration are displayed.
INCLOUD PASS. REFERENCE	Only active if a dye has been defined as a passive reference on the SETTINGS /SCAN project tab If this option is activated, the fluorescence of the dye that has been set as a passive reference is used for standardization.
GROUP	If several experiments were carried out on the PCR plate, select the group of the experiment to be analyzed. (→ Section "Defining groups" p. 41).
THRESHOLD	Manually adjust threshold value The threshold value must be between 1 and 100, depending on the standardized representation of the fluorescence curves (dRn). **Note:** The threshold value can be calculated automatically or set manually in the chart (→ see also "Setting the threshold value" below).
▶	Opens the selection window with display options (see section "Displaying the fluorescence curves for absolute quantification" p. 60).

Setting the threshold value

To determine Ct values for the evaluation, a threshold value needs to be determined for each experiment first.

You have several options for setting the threshold value:

- In the general options (see section "Making basic settings" p. 54).
- Manually in the parameters for the respective evaluation (see table above)
- Graphically in the fluorescence curves representation
- By automatic calculation

❑ In the chart, move the black threshold line up or down with the cursor. Press and hold the left mouse button while doing so. At the same time, the Ct values in the sample table are updated.

Note:
Due to the further spread of the early exponential area of the product accumulation curves, a logarithmic representation is better suited for setting the threshold manually in the display range than a linear representation.

AbsQuant / Autom. Threshold

- ❑ The automatic calculation of the threshold value is activated by clicking on the symbol.
  Alternatively, you can call up the ABSQUANT / AUTOM. THRESHOLD menu command.

Whether you choose manual or automatic calculation, the resulting threshold value is updated and displayed synchronously in the corresponding THRESHOLD input field.

5.2.3 Displaying the fluorescence curves for absolute quantification

In the display range, the measured data, standardized to the value 100 for highest fluorescence intensity, is plotted against the cycle for the selected target gene.

The respective fluorescence curves are displayed by switching to another target gene/dye combination.

The fluorescence data is displayed as a linear or logarithmic representation depending on the selected display option. For both display options the program shows brief information on the sample if the cursor is placed on one of the curves.

Switching the display options for the chart:

1. Click on the button in the parameter bar.
   A selection window for the display options opens.
2. Select the SCALING LOGARITHMIC or LINEAR option.
   Click next to the selection window. The changes are applied.

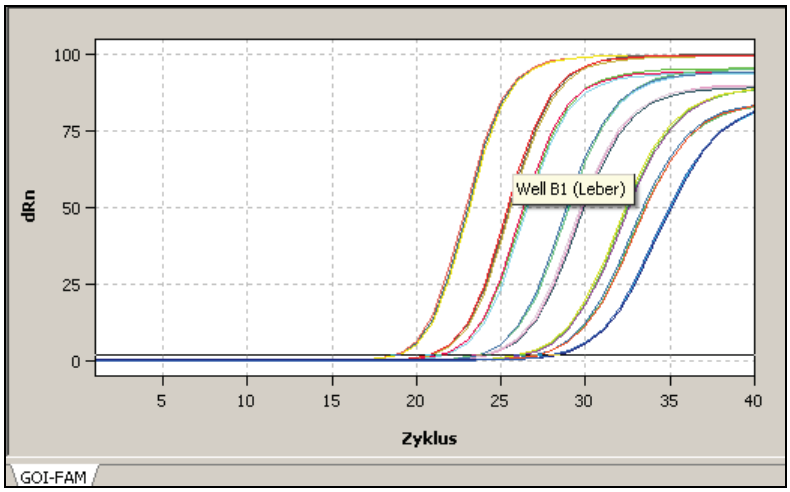


Fig.51 Linear representation of the fluorescence curve for the absolute quantification

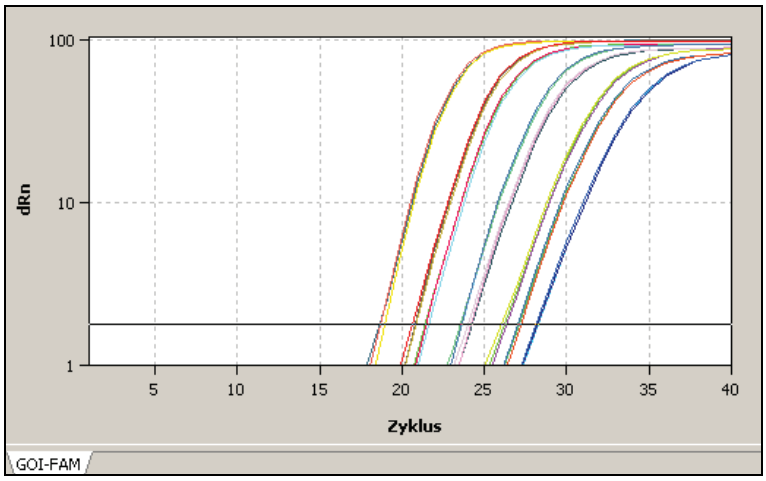


Fig. 52 Logarithmic representation of the fluorescence curve with horizontal threshold line

5.2.4 Displaying the standard curve and results of an absolute quantification

In the bottom STANDARD CURVE section of the ANALYSIS project window, you can switch between the calculated standard curve and the sample table via the CURVE and TABLE list sheets.

For the display of the standard curve the Ct values of the standard samples are plotted graphically against the logarithm of their concentration. In the value range on the right the following calculated data is displayed:

- the coefficient of determination R2 of the linear equation
- the standard curve gradient
- the intersection of the curve with the y-axis at x=0 (offset)
- The PCR efficiency

The standard curve and the values are automatically calculated by the qPCRsoft software and updated in case of settings modifications.

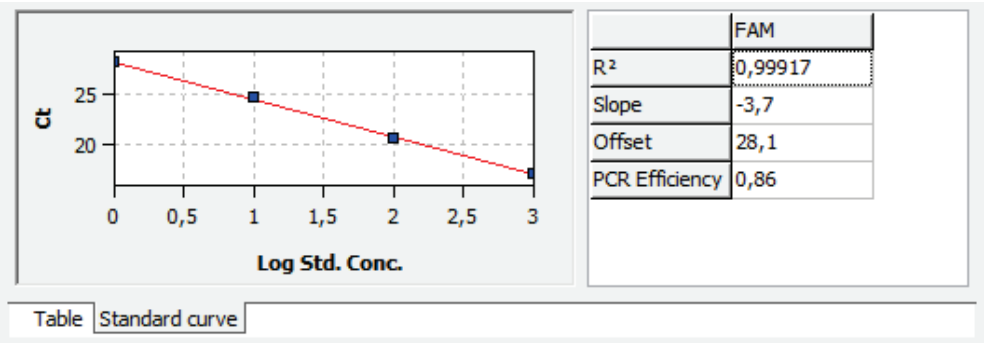


Fig. 53 Standard curve for absolute quantification

The sample table for the absolute quantification contains all data and the associated measurement values for the samples.

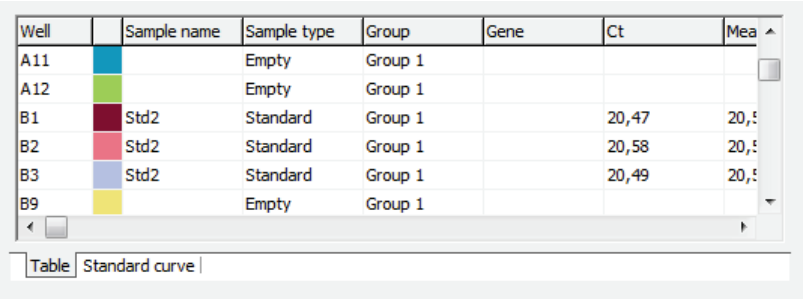


Fig. 54 Sample table for absolute quantification

The selection of the displayed columns can be user-defined. Right-click on a column header to display the selection field with following options:

Column	Description
WELL	Position of sample
COLOR OF CURVE	Each sample is automatically assigned an unchangeable color which is used to display the corresponding fluorescence curve
SAMPLE NAME	Name of sample
SAMPLE TYPE	Type of sample
GROUP	Assignment of the sample to an experimental group
GENE	Name of gene measured in the sample
CT	Ct value of sample
MEAN. CT	Mean Ct value of replicates
CONC. STD	Concentration of the standard sample
MEAN CONC.	Concentration determined from the standard curve on the basis of the mean Ct value
STDDEV.. CT	Standard deviation of the Ct values between replicates
%CV CT	Variation coefficient of the Ct values between replicates

The individual columns can be shown or hidden by selecting or deselecting them. Moreover, the columns can be rearranged freely. To exchange columns, press and hold the left mouse button and drag a column header to the desired location. The display of the results in the table can thus be customized individually.

Display in the project explorer

A shortened representation of the standard curve calculated by the software is displayed in the project explorer under ABS. QUANT. The image displays the graphical plot of the Ct values of the standard samples against the logarithm of their concentration:

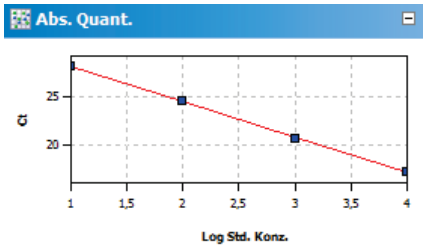


Fig. 55 Representation of the standard curve in the project explorer

5.2.5 Importing the standard curve

Next to the option to measure a standard curve in the experiment, the qPCRsoft software can also be used to determine the concentration of the samples based on the saved standard curve. You can use the import function for this purpose.

AbsQuant / Import standard curve

1. Use the symbol in the toolbar to open the IMPORT STANDARD CURVE window.

   Optionally, you can call up the ABSQUANT / IMPORT STANDARD CURVE menu command.

   The mathematical equation that the standard curve is based on and the associated dye are each displayed in the list fields of the window.

2. Select one of the import options from the IMPORT STANDARD CURVE window and make the corresponding entries:

Option	Meaning
IMPORT FROM THIS RUN	Imports a standard curve from the current open project. If several standard curves are saved in one project, all curves are displayed and you can make a selection.
IMPORT FROM SAVED RUN	Imports a standard curve from a saved project. If several standard curves have been saved, select the corresponding curve from the list.
MANUAL INPUT	Standard curve coefficients are entered manually. Enter the gradient and the intercept for the equation Ct = gradient * log(conc) + intercept.

5.2.6 Deleting the evaluation of an absolute quantification

An evaluation that is no longer required can be removed.

1. Activate the evaluation by selecting its description in the evaluation list of the method tab.

2. Click on in the toolbar.

   The evaluation is removed.

5.3 Relative quantification

Relative quantitation allows for determination of the relative expression level of a gene of interest in relation to one or more reference genes (often housekeeping genes). If one of the samples is denoted as the calibrator , the expression level of that sample is set to unity and the relative expression levels of all the other samples are given in relation to the calibrator sample. For relative quantification standard dilution series are required for the gene of interest as well as for the reference genes.

5.3.1 Creating a new evaluation for a relative quantification

RelQuant / Add rel. quantification

1. Go to the ANALYSIS / RELQUANT project tab.
   If the tab is not visible, click on the arrows in the tab bar. This will scroll the tabs.
2. Click on the symbol in the toolbar.
   Alternatively, call up the RELQUANT / ADD REL. QUANTIFICATIONmenu command.
3. An input window appears. Enter the description for the current evaluation.

   On the REL. QUANT. tab the following information is activated:

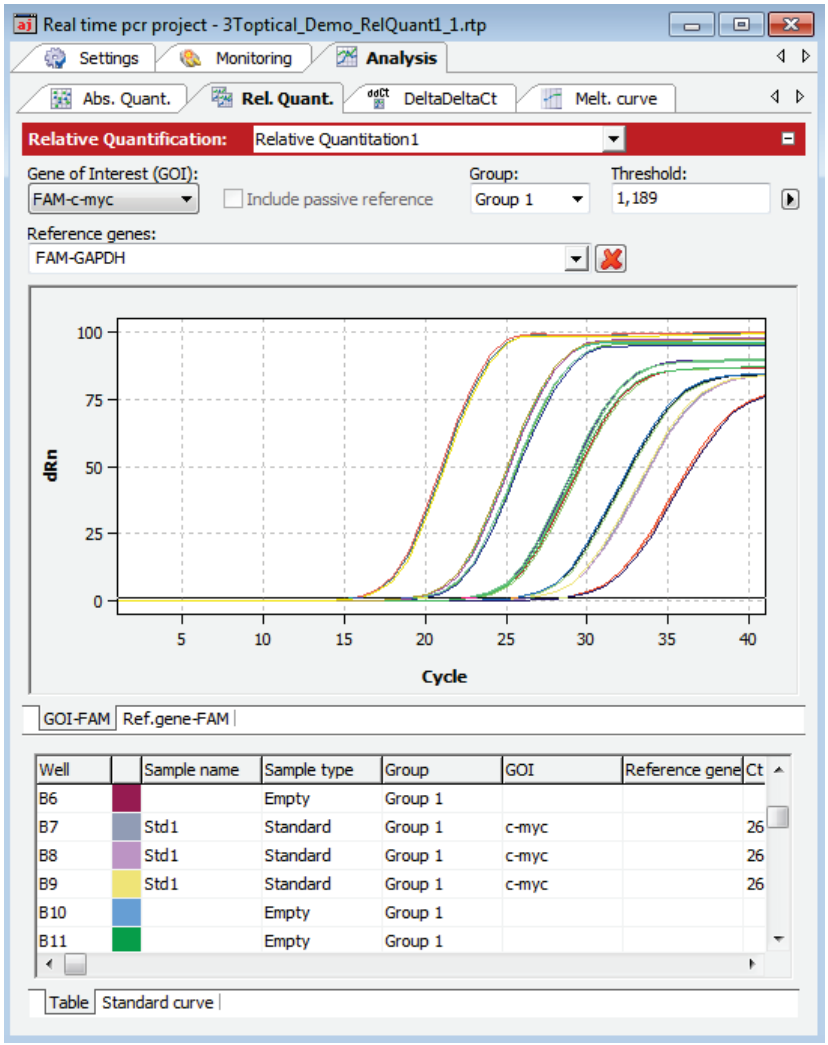


Fig. 56 Window for relative quantification

- Parameter settings
- Display of the fluorescence curves for the target gene and the reference gene
- Display of the standard curves for the target gene and the reference gene and the calculated coefficients
- Display of the sample table with the added values

5.3.2 Parameter settings for relative quantification

Fig. 57 Parameter settings for relative quantification

The following parameters must be set for the relative quantification:

Option	Description
SELECTION LIST	Selection of an evaluation created for the experiment
GENE OF INTEREST (GOI)	Selection list of target gene/dyecombinations According to the selection, the fluorescence and regression curves for the concentration are displayed. Only one target gene at a time can be selected.
REFERENCE GENES	Reference gene selection list In contrast to the target gene, several reference genes can be selected at the same time. The number of list sheets in the display range therefore grows with each reference gene. The symbol is used to remove all reference gene settings from the evaluation.
INCLUDE PASS. REFERENCE	Only active if a dye has been defined as a passive reference on the SETTINGS / SCAN project tab. If this option is activated, the fluorescence of the dye that has been set as a passive reference is used for standardization.
GROUP	If several experiments were carried out on the PCR plate, select the group of the experiment to be analyzed. (→ Section "Defining groups" p. 41).
THRESHOLD	Manually adjust threshold values The threshold value must be between 1 and 100, depending on the standardized representation of the fluorescence curves (dRn). **Note:** The threshold value can be calculated automatically or set in the graph.
	Opens the selection window with display options

Setting the threshold value

To determine Ct values for the evaluation, a threshold value needs to be determined for each experiment first.

You have several options for setting the threshold value:

- In the general options (see section "Making basic settings" p. 54)
- Manually in the parameters for the respective evaluation (see table above)
- Graphically in the fluorescence curves representation
- By having it calculated automatically

❑ In the chart, move the black threshold line up or down with the cursor. Press and hold the left mouse button while doing so. At the same time, the Ct values in the sample table are updated.

Note:
Due to the further spread of the early exponential area of the product accumulation curves, a logarithmic representation is better suited for setting the threshold manually in the display range than a linear representation.

RelQuant / Autom. Threshold

❑ The automatic calculation of the threshold value is activated by clicking on the symbol.
Alternatively, you can call up THE RELQUANT / AUTOM. THRESHOLD menu command.

Whether you choose manual or automatic calculation, the resulting threshold value is updated and displayed synchronously in the corresponding THRESHOLDinput field.

5.3.3 Displaying the fluorescence curves in the relative quantification

In the display range, the measured data, standardized to the value 100 for highest fluorescence intensity, is plotted against the cycle for the selected target gene. The target gene/dye and the reference gene/dye combinations are each assigned a list sheet that can be activated by clicking on the gene/dye tab on the bottom.

Since only one target gene/dye combination is permitted at a time, the fluorescence curves of the selected combination are displayed whenever a new combination is selected. The number of the available list sheets depends on the number of the selected reference genes.

The fluorescence data is displayed as a linear or logarithmic representation depending on the selected display option. For both display options the program shows brief information on the sample if the cursor is placed on one of the curves.

Switching the display options for the chart:

1. Click on the button in the parameter bar.
   A selection window for the display options opens.
2. Select the SCALING LOGARITHMIC or LINEAR option.
   Click next to the selection window. The changes are applied.

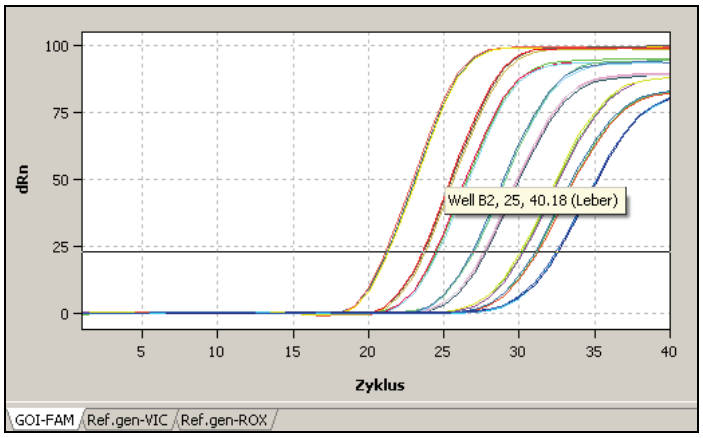


Fig.58 Linear representation of the fluorescence curve for the relative quantification

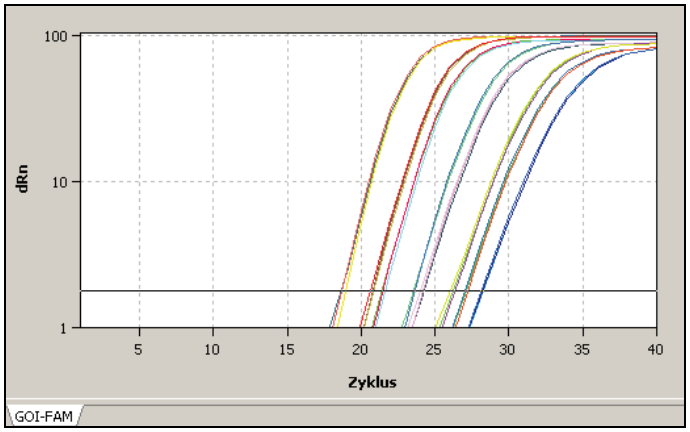


Fig.59 Logarithmic representation of the fluorescence curve for the relative quantification with horizontal threshold line

5.3.4 Displaying the standard curves and the results of a relative quantification

In the upper area of the project window you can switch between the display of the calculated standard curves for the target gene and all selected reference genes and the sample table for the relative quantification using the STANDARD CURVE and TABLE list sheets.

For the display of the standard curve the Ct values of the standard samples are plotted graphically against the logarithm of their concentration. In the value range on the right the following calculated data is displayed:

- the coefficients of determination R^2 of the linear equation
- the standard curve gradients
- the intersections of the curves with the y-axis at x=0 (offset)
- The PCR efficiency

The standard curve and the values are automatically calculated by the qPCRsoft software and updated in case of settings modifications. According to the number of genes used a scroll bar appears under the table. It can be used to navigate through the table's columns.

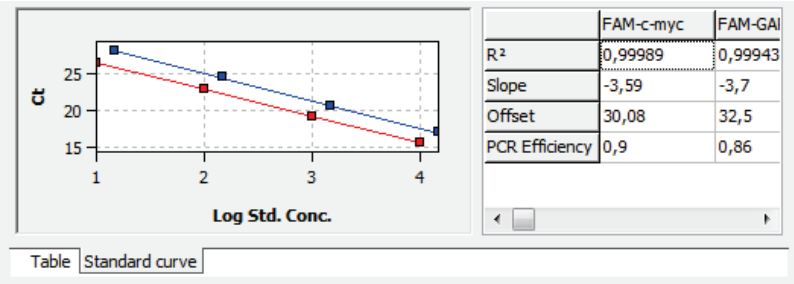


Fig.60 Standard curves for relative quantification

The sample table for the relative quantification contains all data and the associated measurement values for the samples.

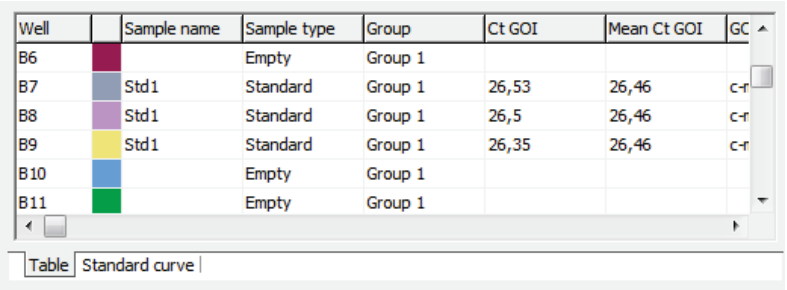


Fig.61 Sample table for relative quantification

The selection of the displayed columns can be user-defined. Right-click on a column header to display the following selection field:

Column	Meaning
WELL	Position of sample
COLOR OF CURVE	Each sample is automatically assigned an unchangeable color which is used to display the corresponding fluorescence curve
SAMPLE NAME	Name of sample
SAMPLE TYPE	Type of sample
GROUP	Assignment of the sample to an experimental group
GOI	Gene of interest
REFERENCE GENE	Reference gene
CT GOI	Ct value of gene of interest
CT REFERENCE GENE	Ct value of reference gene
MEAN. CT GOI	Mean Ct value of replicates of the gene of interest
MEAN. CT REF.GEN	Mean Ct value of replicates of the reference gene
CONC. STD. GOI	Concentration of the standard for the gene of interest
CONC. STD REF.GEN	Concentration of the standard for the reference gene
MEAN CONC. GOI	Concentration for the gene of interest determined from the standard curve on the basis of the mean Ct value
MEAN CONC.. REF. GEN	Concentration for the reference gene determined from the standard curve on the basis of the mean Ct value
STD.DEV. CT GOI	Standard deviation of the Ct values between replicates of the gene of interest
STD.DEV. REFGEN	Standard deviation of the Ct values between replicates of the reference gene

%CV CT GOI	Variation coefficient of the Ct values between replicates of the gene of interest
%CV CT REFGEN	Variation coefficient of the Ct values between replicates of the reference gene
RELATIVE CONC.	Relative (x-fold) expression level of the gene of interest in relation to the reference gene
NORM. REL. CONC.	Relative (x-fold) expression level of the gene of interest in relation to the reference gene, standardized to the expression of the calibrator (if defined)

The individual columns can be shown or hidden by selecting or deselecting them. Moreover, the columns can be rearranged freely. To exchange to columns, press and hold the left mouse button and drag a column header to the desired location. The display of the results in the table can thus be customized individually.

Display in the project explorer

A shortened representation of the standard curves calculated by the software is displayed in the project explorer under RELATIVE QUANTIFICATION. The image displays the graphical plot of the Ct values of the standard samples against the logarithm of their concentration.

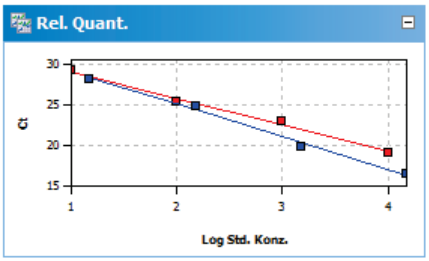


Fig.62 Representation of the standard curve in the project explorer

5.3.5 Importing the standard curve for relative quantification

Next to the option to measure a standard curve in the experiment, the qPCRsoft software can also be used to determine the concentration of the samples based on the saved standard curve. You can use the import function for this purpose.

1. Use the symbol in the toolbar to open the IMPORT STANDARD CURVE window.

   RelQuant / Import standard curve

   Optionally, you can call up the RELQUANT / IMPORT STANDARD CURVE menu command.

2. All additional setting are analogous to the settings for the absolute quantification (→ see section "Importing the standard curve" S. 63).

5.3.6 Deleting the evaluation of a relative quantification

An evaluation that is no longer required can be removed.

RelQuant / Delete rel. quantification

1. Activate the evaluation by selecting its description in the evaluation list of the method tab.

2. Click on the symbol in the toolbar.
   Alternatively, call up the RELQUANT / DELETE REL. QUANTIFICATION menu command.

   The evaluation is removed.

5.4 ΔΔCt method

The ΔΔCt-method allows for determination of the relative expression level of a gene of interest in relation to one or more reference genes (often housekeeping genes). One of the samples has to be denoted as the calibrator. The expression level ofthe calibrator is set to unity and the relative expression levels of all the other samples are given in relation to the calibrator sample. In ΔΔCt-method there is no need to measure standard dilution series. However, if the ΔΔCt-method shall be validated within the same PCR run standard dilution series must be defined.

5.4.1 Creating a new evaluation for a ΔΔCt method

DeltaDeltaCt / Add ΔΔCt Quantification

1. Go to the ANALYSIS / RELQUANT project tab.
   If the tab is not visible, click on the arrows in the tab bar. This will scroll the tabs.
2. Click on the ddCt symbol in the toolbar.
   Alternatively, call up the DELTADELTACT / ADD DDCT QUANTIFICATION menu command.
3. An input window appears. Enter the description for the current evaluation.

   On the DELTADELTACT tab the following information is activated:

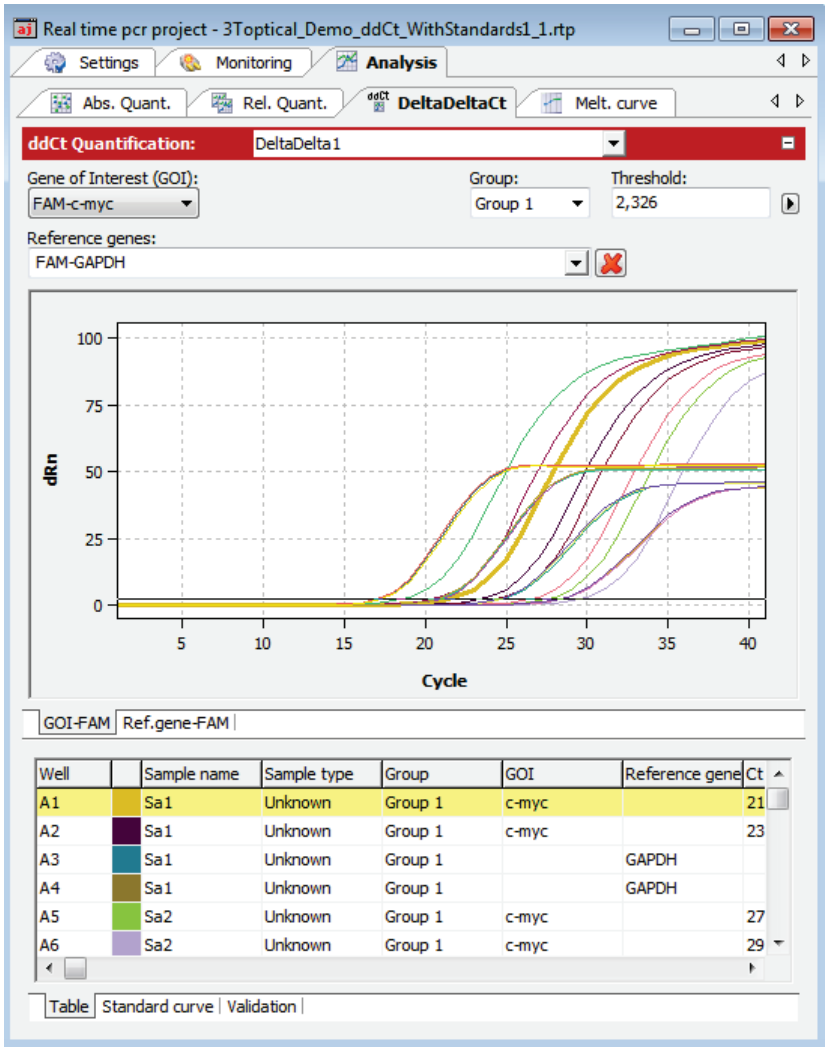


Fig.63 Project window for the ΔΔCt method

- Parameter settings
- Display of the fluorescence curves for the target gene and the reference gene
- If standards have been defined, display of the standard curve and the validation curve for the expression level of the gene of interest in relation to the reference gene and the calculated factors
- Display of the sample table with the added values

5.4.2 Parameter settings for the ΔΔCt method

Fig.64 Parameter settings for the ΔΔCt method

The following parameters must be set for the ΔΔCt method:

Option	Description
SELECTION LIST	Selection of an evaluation created for the experiment
GENE OF INTEREST (GOI)	Selection list of combinations of measured dyes and target genes to be quantified Only one target gene at a time can be selected.
REFERENCE GENES	Selection list for the reference genes In contrast to the target gene, several reference genes can be selected at the same time. The number of list sheets in the display range therefore grows with each reference gene. With the symbol you can remove all selected reference genes from the selection.
GROUP	If several experiments were carried out on the PCR plate, select the group of the experiment to be analyzed. (→ Section "Defining groups" p. 41).
THRESHOLD	Manually adjust threshold values The threshold value must be between 1 and 100, depending on the standardized representation of the fluorescence curves (dRn). **Note:** The threshold value can be calculated automatically or set in the graph.
	Opens the selection window with display options

Setting the threshold value

To determine Ct values for the evaluation, a threshold value needs to be determined for each experiment first.

You have several options for setting the threshold value:

- In the general options (see section "Making basic settings" p. 54).
- Manually in the parameters for the respective evaluation (see table above)
- Graphically in the fluorescence curves representation
- By having it calculated automatically

❑ In the chart, move the black threshold line up or down with the cursor. Press and hold the left mouse button while doing so. At the same time, the Ct values in the sample table are updated.

Note:
Due to the further spread of the early exponential area of the product accumulation curves, a logarithmic representation is better suited for setting the threshold manually in the display range than a linear representation.

DeltaDeltaCt / Autom. Threshold

❑ The automatic calculation of the threshold value is activated by clicking on the symbol.
Optionally, you can call up the DELTADELTACT / AUTOM. THRESHOLD menu command.

Whether you choose manual or automatic calculation, the resulting threshold value is updated and displayed synchronously in the corresponding THRESHOLDinput field.

5.4.3 Displaying the fluorescence curves for the ΔΔCt method

In the display range, the measured data, standardized to the value 100 for highest fluorescence intensity, is plotted against the cycle for the selected target gene. The gene/dye and the reference gene/dye combinations are each assigned a list sheet that can be activated by clicking on the gene/dye tab on the bottom.

In the evaluation you can always use only one target gene but several reference genes. The number of the available list sheets depends on the number of the selected genes.

The fluorescence data is displayed as a linear or logarithmic representation depending on the selected display option. For both display options the program shows brief information on the sample if the cursor is placed on one of the curves.

Switching the display options for the chart:

1. Click on the button in the parameter bar.

   A selection window for the display options opens.

2. Select the SCALING LOGARITHMIC or LINEAR option.

   Click next to the selection window. The changes are applied.

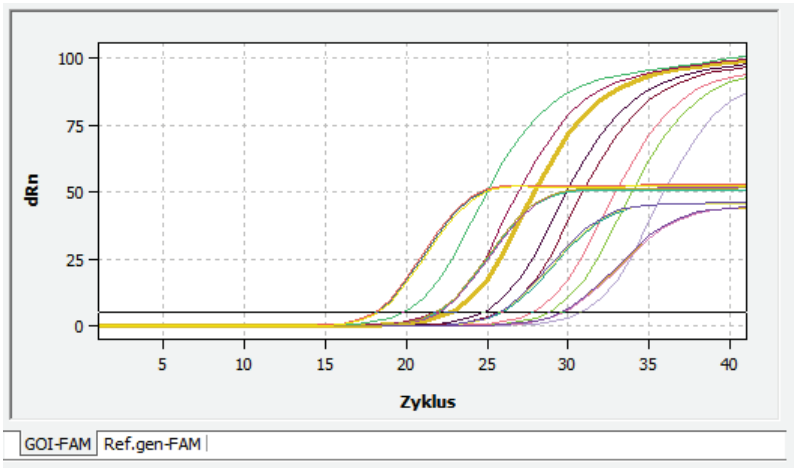


Fig.65 Linear representation of the fluorescence curve for the ΔΔCt method

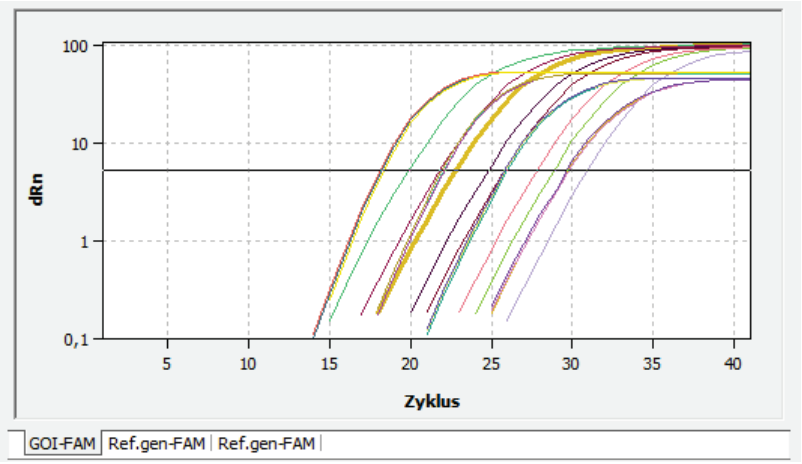


Fig.66 Logarithmic representation of the fluorescence curve for the ΔΔCt method

5.4.4 Setting the calculation mode for the standardized expression

The qPCRsoft software enables you to calculate the standardized expression SE with two different methods:

- Without PCR efficiency calculation (Livak method)
- With PCR efficiency calculation for GOI and reference genes (Pfaffl method)

To calculate the standardized expression SE, one sample must be defined as the calibrator.

Calculating the standardized expression SE without efficiency calculation (Livak method)

The Livak method assumes that the PCR efficiencies of the gene of interest (GOI) and the reference gene (RefG) are equal. The following applies:

$$NE = 2^{-\Delta\Delta Ct}$$

where $\Delta\Delta Ct = \Delta Ct(Calibrator) - \Delta Ct(Sample)$

and $\Delta Ct(Sample) = Ct(GOI, Sample) - Ct(RefGene, Sample)$

$\Delta Ct(Calibrator) = Ct(GOI, Calibrator) - Ct(RefGene, Calibrator)$

Calculating the standardized expression SE with efficiency calculation (Pfaffl method)

The Pfaffl method considers the efficiencies determined for the gene of interest (GOI) and the reference gene (RefGene). The efficiencies (E(GOI) and E(RefGene)) can be calculated from dilution series or specified in the software. The following applies:

$$NE = \frac{[E(GOI)]^{\Delta Ct(GOI)}}{[E(RefGene)]^{\Delta Ct(RefGene)}}$$

where $\Delta Ct(GOI) = Ct(GOI, Calibrator) - Ct(GOI, Sample)$

and $\Delta Ct(RefGene) = Ct(RefGene, Calibrator) - Ct(RefGene, Sample)$

The Pfaffl method is generally preferred as the basic assumption of the Livak method (equal efficiency when amplifying the gene of interest and the reference gene) is rarely the case in practice and the calculation can therefore lead to incorrect values.

The calculation method is selected in the DDCT OPTIONS window.

- ❑ Click on the symbol in the toolbar.
  Alternatively, call up the DELTADELTACT / DDCT QUANT. OPTIONS menu command.
- ❑ Select the desired calculation method.
  For the Pfaffl method, the efficiency values can be determined automatically from the standard curves (dilution series) for the gene of interest and the reference gene (if standards have been defined) or the values can be entered manually in the respective fields.

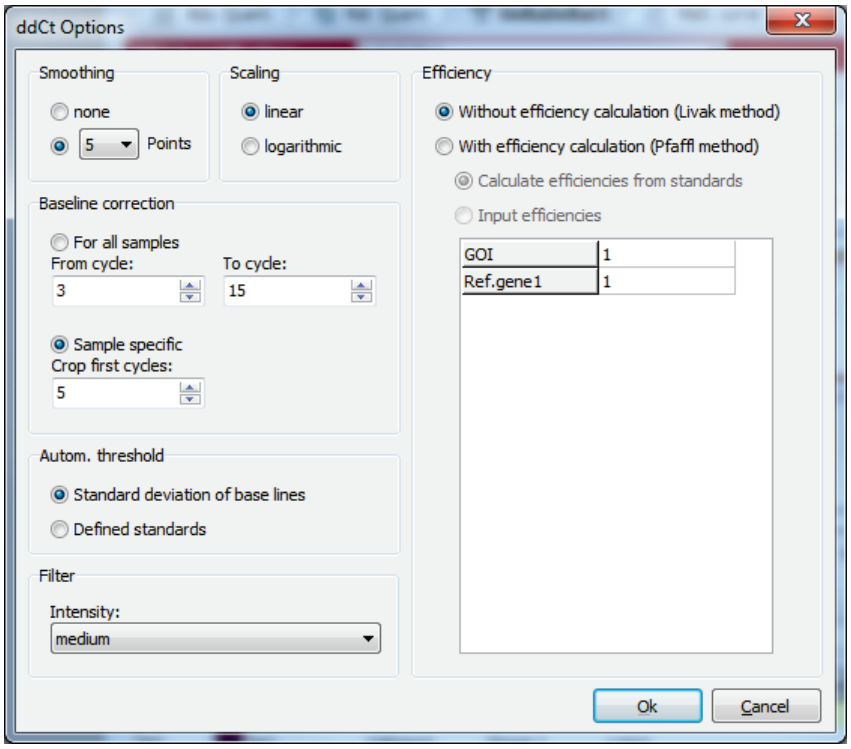


Fig. 67 DDCT OPTIONS window for presetting the analysis of the ΔΔCt method

5.4.5 Displaying the validation curves and values

To calculate the ΔΔCt values there is no need to determine a validation curve. However, it might be useful to consult it when checking the data quality.Prerequisite for creating a validation curve is measuring a standard series with different dilution levels of target gene and reference gene. If standard series have been measured for the target and reference gene, the expression ratio between target and reference gene is represented graphically in the VALIDATION CURVE display. For this purpose, the mean Ct value of the target gene is subtracted from the mean Ct value of the reference gene for the corresponding dilution level and the resulting dCt(V) value is plotted against the logarithm of the concentration.

In the value range on the right the following calculated data is displayed:

- The coefficient of determination R^2
- The gradient of the validation straight line
- the intersection of the curve with the y-axis at x=0 (offset)

The gradient of the curve should not exceed a value of ± 0.1. The assumption then applies that the efficiencies of the amplification of the gene of interest and the reference gene are more or less identical and the calculation of the ΔΔCt values produces valid data.

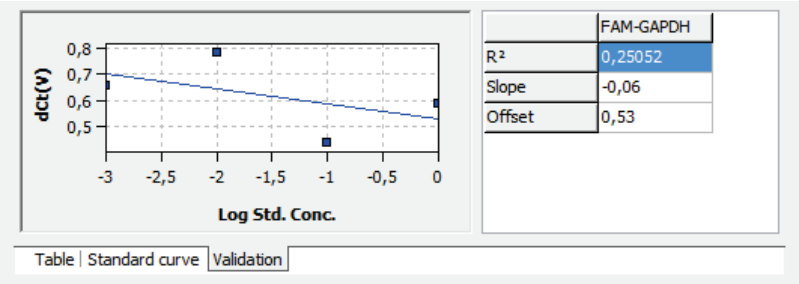


Fig. 68 Validation curves view for the ΔΔCt method

You can switch between the ΔΔCt calculation views using the Table, Standard curve and Validation tabs. The validation curves and the values are automatically calculated by the qPCRsoft software and updated if the settings change.

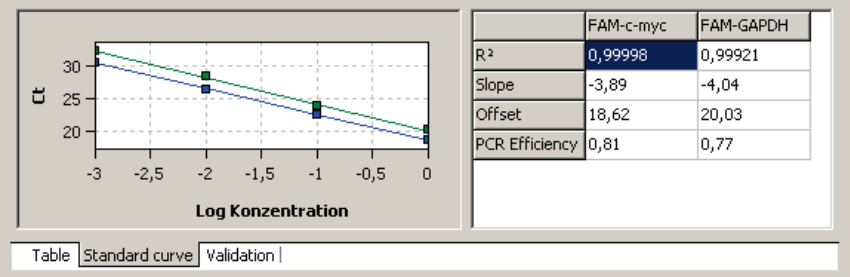


Fig. 69 Validation curves view for the ΔΔCt method

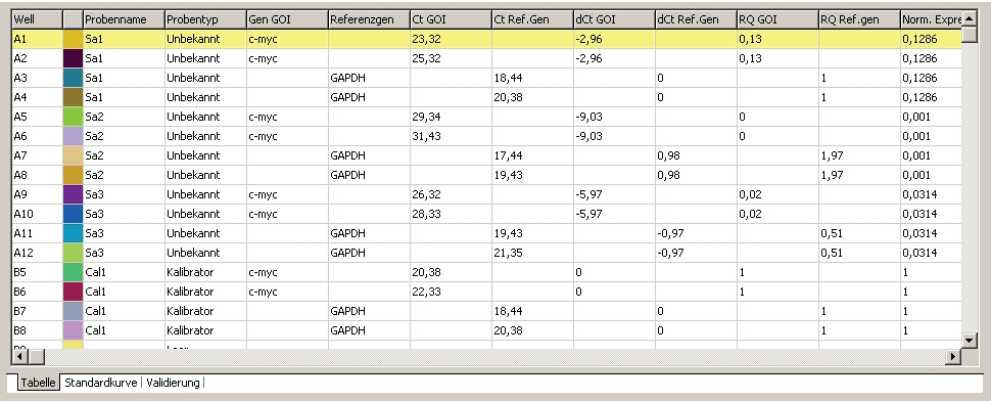

Well
Probenname
Probentyp
Gen GOI
Referenzgen
Ct GOI
Ct Ref.Gen
dCt GOI
dCt Ref.Gen
RQ GOI
RQ Ref.gen
Norm. Expre
A1 Sa1 Unbekannt c-myc 23,32 -2,96 0,13 0,1286
A2 Sa1 Unbekannt c-myc 25,32 -2,96 0,13 0,1286
A3 Sa1 Unbekannt GAPDH 18,44 0 1 0,1286
A4 Sa1 Unbekannt GAPDH 20,38 0 1 0,1286
A5 Sa2 Unbekannt c-myc 29,34 -9,03 0 0,001
A6 Sa2 Unbekannt c-myc 31,43 -9,03 0 0,001
A7 Sa2 Unbekannt GAPDH 17,44 0,98 1,97 0,001
A8 Sa2 Unbekannt GAPDH 19,43 0,98 1,97 0,001
A9 Sa3 Unbekannt c-myc 26,32 -5,97 0,02 0,0314
A10 Sa3 Unbekannt c-myc 28,33 -5,97 0,02 0,0314
A11 Sa3 Unbekannt GAPDH 19,43 -0,97 0,51 0,0314
A12 Sa3 Unbekannt GAPDH 21,35 -0,97 0,51 0,0314
B5 Cal1 Kalibrator c-myc 20,38 0 1 1
B6 Cal1 Kalibrator c-myc 22,33 0 1 1
B7 Cal1 Kalibrator GAPDH 18,44 0 1 1
B8 Cal1 Kalibrator GAPDH 20,38 0 1 1
Tabelle
Standardkurve
Validierung

Fig. 70 Sample table view for the ΔΔCt method

The selection of the displayed columns can be user-defined. Right-click on a column header to display the selection field with following options:

Column	Meaning
WELL	Position of sample*
COLOR OF CURVE	Each sample is automatically assigned an unchangeable color which is used to display the corresponding fluorescence curve
SAMPLE NAME	Name of sample*

SAMPLE TYPE	Type of sample*
GROUP	Assignment of the sample to an experimental group*
GOI	Gene of interest
REFERENCE GENE	Reference gene
CT GOI	Ct value of gene of interest
CT REF.GENE	Ct value of reference gene
MEAN CT GOI	Mean Ct value of replicates of the gene of interest
MEAN CT REF.GENE	Mean Ct value of replicates of the reference gene
STD.DEV. CT GOI	Standard deviation of the Ct values between replicates of the gene of interest
STD.DEV. CT REF.GENE	Standard deviation of the Ct values between replicates of the reference gene
%CV CT GOI	Variation coefficient of the Ct values between replicates of the gene of interest
%CV CT REF.GENE	Variation coefficient of the Ct values between replicates of the reference gene
DCT GOI	Delta Ct value for replicates of the gene of interest
DCT REF.GENE	Delta Ct value for replicates of the reference gene
RQ GOI	Calculated relative amount for replicates of the gene of interest in the original sample
RQ REF.GENE	Calculated relative amount for replicates of the reference gene in the original sample
MEAN RQ REF.GENE	Average calculated relative amount for replicates of the reference gene in the original sample
NORM. EXPRESSION	Standardized relative (x-fold) expression level of the gene of interest in the sample in relation to the calibrator

The individual columns can be shown or hidden by selecting or deselecting them. Moreover, the columns can be rearranged freely. To exchange to columns, press and hold the left mouse button and drag a column header to the desired location. The display of the results in the table can thus be customized individually.

Display in the project explorer

A shortened representation of the validation curves calculated by the software is displayed in the project explorer under DELTADELTACT. The image displays the graphical plot of the dCt(V) values against the logarithm of the sample concentration:

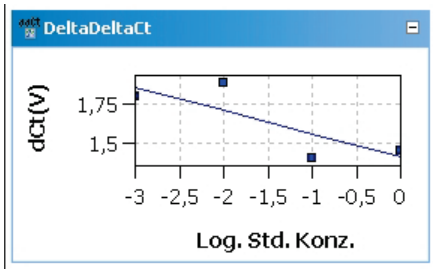


Fig. 71 Representation of the validation curves in the project explorer

5.4.6 Deleting a ΔΔCt method evaluation

An evaluation that is no longer required can be removed.

DeltaDeltaCt / Delete ddCt quantification

1. Activate the evaluation by selecting its description in the evaluation list of the method tab.
2. Click on the

symbol in the toolbar.
Optionally, call up the DELTADELTACT / DELETE DDCT QUANTIFICATION menu command.

The evaluation is removed.

5.5 Melting curve analysis

When performing a melting curve analysis the temperature in the reaction mixture is increased successively until the PCR product is denatured. The dissociation of the fragment in single strands will result in the release of a intercalating dye. The associated reduction of the fluorescence intensity is measured and recorded by the device. By forming the first derivative of the fluorescence curve you will get a peak that describes the melting point and the approximate concentration of the PCR fragment. By means of melting curve analysis you can differentiate whether the reaction has caused the formation of a specific PCR product or whether unspecific by-products, such as primerdimers, were produced.

5.5.1 Creating a new melting curve analysis

Melting curve / Add Melting curve

1. Go to the ANALYSIS / MELT. CURVE project tab.
   If the tab is not visible, click on the arrows in the tab bar. This will scroll the tabs.
2. Click on the symbol in the toolbar.
   Optionally, call up the MELTING CURVE / ADD MELTING CURVEmenu command.
3. An input window appears. Enter the description for the current evaluation.

   On the MELTCURVE tab the following information is activated:

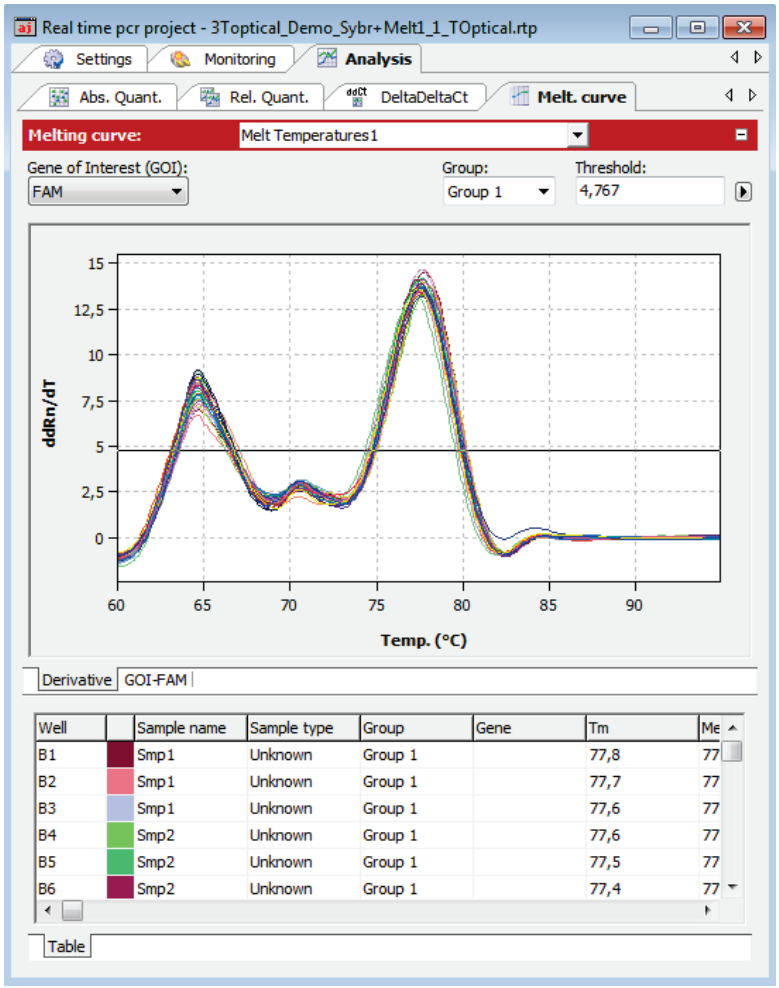


Fig. 72 Window for melting curve analysis

- Parameter settings
- Display of the fluorescence values as a function of the temperature and/or its first derivative
- Display of the sample table

5.5.2 Parameter settings for melting curve analysis

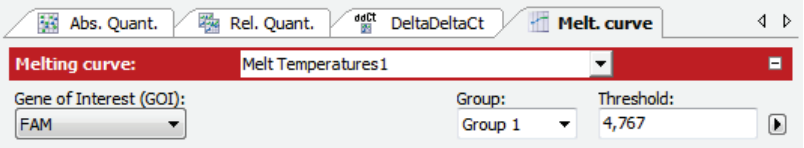


Fig. 73 Parameter settings for melting curve quantification

Set the following parameters for the meltings curve quantification:

Option	Description
SELECTION LIST	Selection of a selection created for the experiment
GENE OF INTEREST (GOI)	Selection list of target gene/ dye combinations Generally, an intercalating dye must be selected for the target gene for the melting curve analysis. According to the selection, the fluorescence and regression curves for the concentration are displayed.
GROUP	If several experiments were carried out on the PCR plate, select the group of the experiment to be analyzed. (→ Section "Defining groups" p. 41).
THRESHOLD	Manually adjust threshold values The threshold is now effective on the DERIVATIVE tab. Only curves whose maximum dRn/dT is greater than the threshold are analyzed. **Note:** The threshold value can be calculated automatically or set manually in the chart (→ see also "Setting the threshold value" below).
▶	Opens the selection window with display options

Setting the threshold value

For the correct evaluation a threshold value must be determined for the melting curves.

You have several options for setting the threshold value:

- In the general options (see section "Making basic settings" p. 54)
- Manually in the parameters for the respective evaluation (see table above)
- Graphically in the representation of the fluorescence curves derivative
- Automatic calculation

Melting curve / Autom. Threshold

❑ In the chartDERIVATIVE, move the black threshold line up or down with the cursor. Press and hold the left mouse button while doing so. At the same time, the Tm values in the sample table are updated.

❑ The automatic calculation of the threshold value is activated by clicking on the symbol.

Optionally, you can call up the MELTING CURVE / AUTOM. THRESHOLD menu command.

Whether you choose manual or automatic calculation, the resulting threshold value is updated and displayed synchronously in the corresponding THRESHOLDinput field.

5.5.3 Displaying fluorescence curves/melting curves

In the display area, the measured fluorescence curves are shown in relation to the temperature and are either standardized to the highest fluorescence value or both standardized to a target value of 100, depending on the settings made in the MELT. CURVE OPTIONS window. The data is displayed as a linear or logarithmic representation depending on the selected display option.

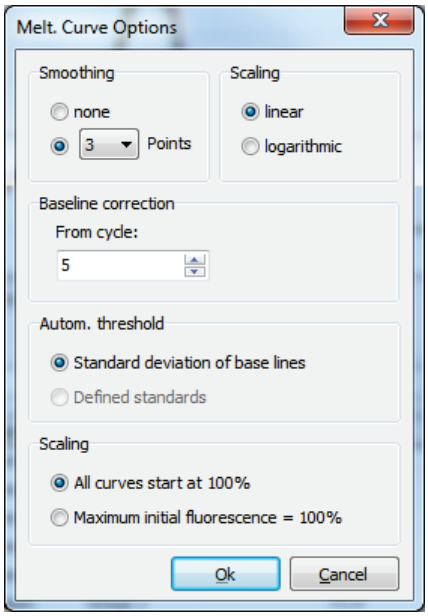


Fig. 74 Display options for melting curve analysis

The program shows brief information on the sample if the cursor is placed on one of the curves.

The melting temperature Tm is determined by forming the first derivative of the melting curves from the maxima of the forming peaks.

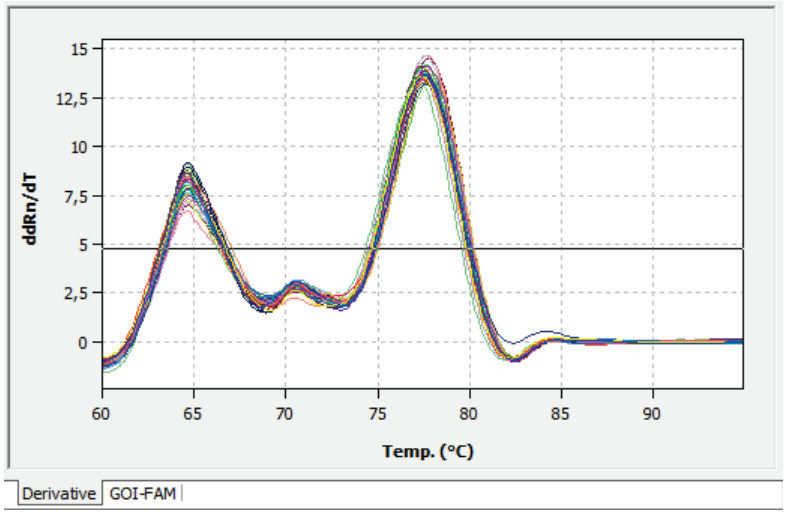


Fig. 75 View of the melting curves derivative with threshold line

You can switch between the melting curves display and the derivatives via the tab in the bottom left corner of the display range.

Switching the display options for the chart of fluorescence curves:

1. Click on the button in the parameter bar.

   A selection window for the display options opens.

2. Select the SCALING LOGARITHMIC or LINEARoption.

   Click next to the selection window. The changes are applied.
   The DERIVATIVE tab cannot be displayed logarithmically.

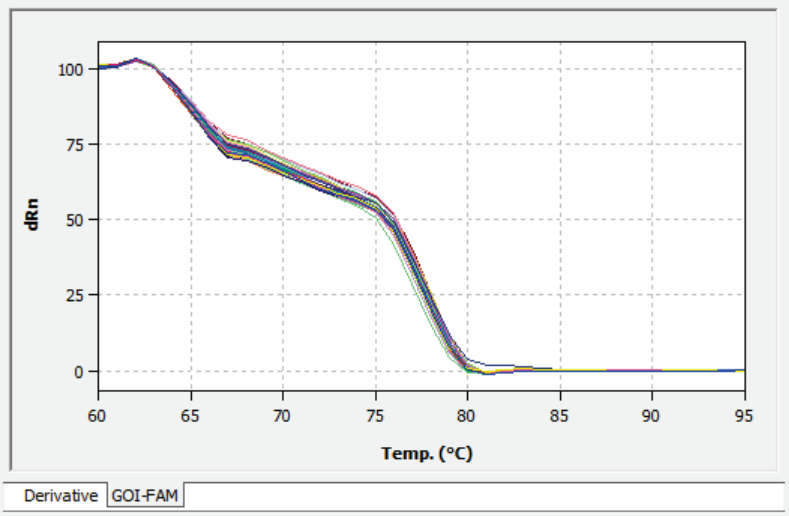
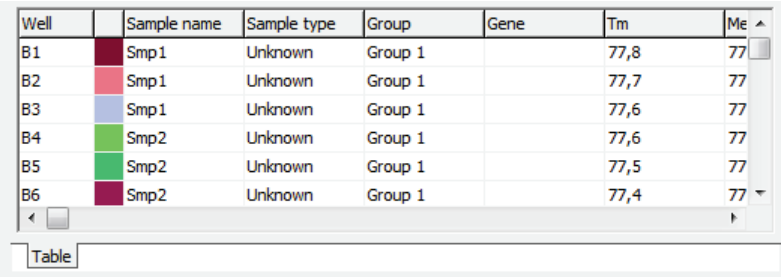


Fig.76 Fluorescence curves display (linear view)

The linear display does not show a threshold line.

5.5.4 Displaying the sample table for the melting curves

The sample table for melting curves contains all data and the associated measurement values for the samples.



Well		Sample name	Sample type	Group	Gene	Tm	Me
B1		Smp1	Unknown	Group 1		77,8	77
B2		Smp1	Unknown	Group 1		77,7	77
B3		Smp1	Unknown	Group 1		77,6	77
B4		Smp2	Unknown	Group 1		77,6	77
B5		Smp2	Unknown	Group 1		77,5	77
B6		Smp2	Unknown	Group 1		77,4	77

Table

Fig.77 Sample table for melting curve analysis

The selection of the displayed columns can be user-defined. Right-click on a column header to display a selection field with following options:

Column	Meaning
WELL	Position of sample
COLOR OF CURVE	Each sample is automatically assigned an unchangeable color which is used to display the corresponding fluorescence curve
SAMPLE NAME	Name of sample

SAMPLE TYPE	Type of sample
GROUP	Assignment of the sample to an experimental group
GEN E	Name of gene measured in the sample
TM	Melting temperature of the sample
MEAN TM	Mean melting temperature of replicates

The individual columns can be shown or hidden by selecting or deselecting them. Moreover, the columns can be rearranged freely. To exchange to columns, press and hold the left mouse button and drag a column header to the desired location. The display of the results in the table can thus be customized individually.

Display in the project explorer

A shortened representation of the melting curves calculated by the software is displayed in the project explorer under "Melt Curve". This representation shows the graphical plot of the first derivative of the fluorescence values against the temperature.

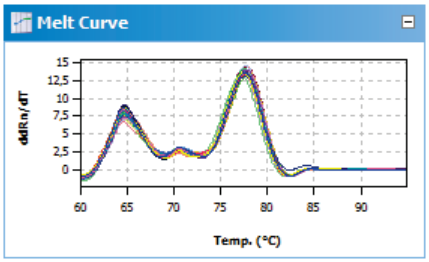


Fig. 78 Representation of the melting curve derivatives in the project explorer

5.5.5 Deleting a melting curve analysis

A melting curve analysis that is no longer required can be removed.

Melting curve / Delete melting curve

1. Activate the evaluation by selecting its description in the evaluation list of the method tab.
2. Click on the symbol in the toolbar.
   Alternatively, call up the MELTING CURVE / DELETE MELTING CURVE menu command.

   The evaluation is removed.

5.6 Genotyping

Genotyping serves to determine sequence differences between a sample and a standard. The standard is defined as the reference sequence (wild type); the genetic condition of the sample is to be determined in the experiment. Genotyping shows which alleles an individual has inherited from its parents.

5.6.1 Creating a new evaluation for genotyping

Genotyping / Add genotyping

1. Go to the ANALYSIS / GENOTYPING project tab.
   If the tab is not visible, click on the arrows in the tab bar. This will scroll the tabs.
2. Click on the symbol in the toolbar.
   Alternatively, call up the GENOTYPING / ADD GENOTYPING menu command.
3. An input window appears. Enter the description for the current evaluation.

The following information is activated on the GENOTYPING tab:

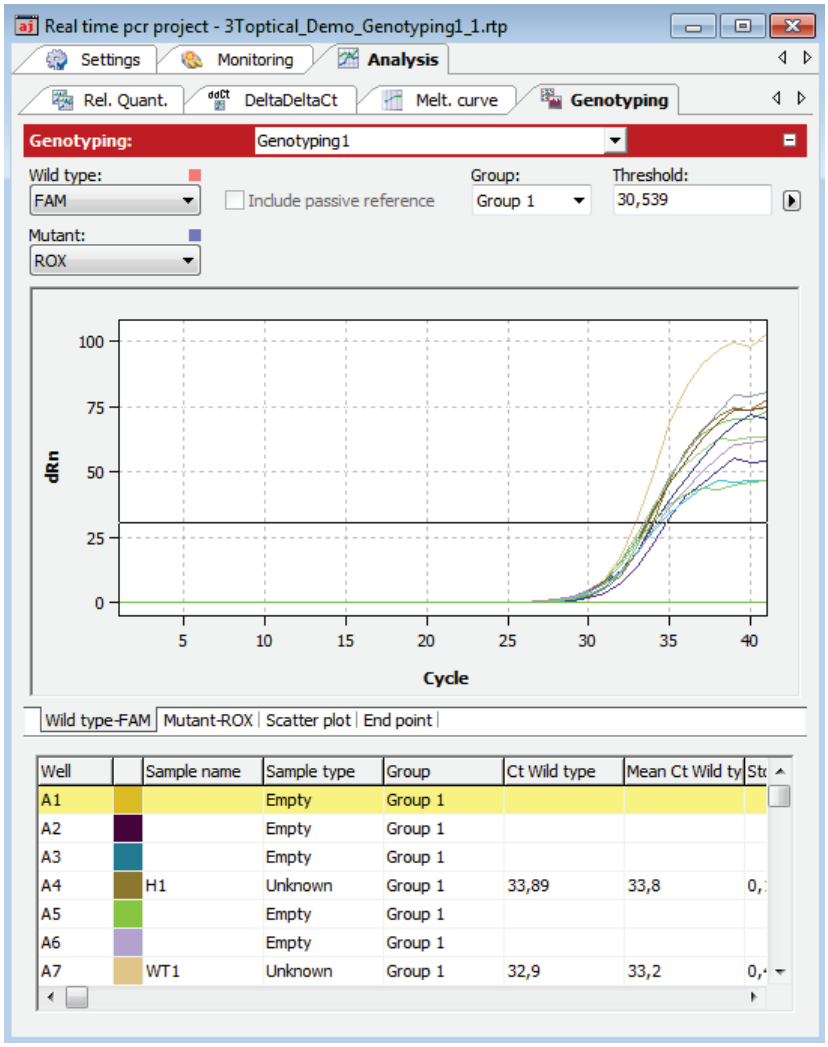


Fig. 79 Genotyping window

- Parameter setting for defining the threshold and selecting the dye with which the wild type and the mutant were measured

- Display of the fluorescence curves and/or display of the results as a scatter plot or bar graph
- Display of the sample table with the results

5.6.2 Parameter settings for genotyping

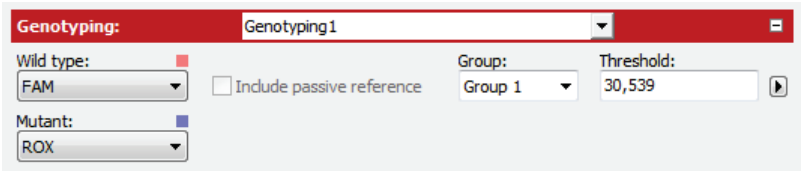


Fig. 80 Parameter settings for genotyping for the fluorescence curve display

Set the following parameters for the absolute quantification:

Option	Description
SELECTION LIST	Selection of an evaluation created for the experiment
WILD TYPE	Selection list for the dye with which the wild type was measured.
MUTANT	Selection list for the dye with which the mutant was measured.
INCLUDE PASSIVE REFERENCE	Only active if a dye has been defined as a passive reference on the SETTINGS / SCAN project tab. If this option is activated, the fluorescence of the dye that has been set as a passive reference is used for standardization.
GROUP	If several experiments were carried out on the PCR plate, select the group of the experiment to be analyzed. (→ "Defining groups", page 41).
THRESHOLD	Manually adjust threshold value. The threshold value must be between 1 and 100, depending on the standardized representation of the fluorescence curves (dRn). *Note*: The threshold value can be calculated automatically or set manually in the chart (→ see also "Setting the threshold value" below).
▶	Opens the selection window with display options (see "Displaying the fluorescence curves for absolute quantification" on page 60).

When changing to the scatter plot or end point bar graph, two fields are displayed for the CUTOFF value for the WILD TYPE and the mutant instead of the Threshold field:

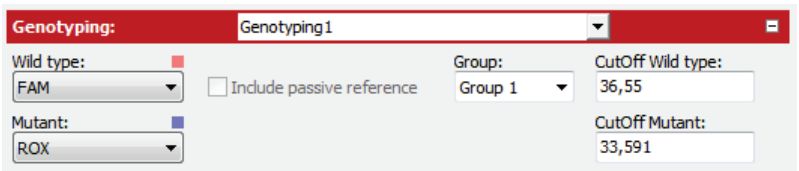


Fig.81 Parameter settings for the scatter plot and the bar graph of a genotyping

Setting the threshold value

To determine Ct values for the evaluation a threshold value must be determined for each experiment.

You have several options for setting the threshold value:

- in the general options (see "Making basic settings" on page 54)
- manually in the parameters for the respective evaluation (see table above)
- graphically in the fluorescence curves representation
- by having it calculated automatically

❑ In the chart, move the black threshold line up or down with the cursor. Press and hold the left mouse button while doing so. At the same time, the Ct values in the sample table are updated.

Note:
The logarithmic view is more suitable for placing the threshold manually in the display area than the linear view because of the wider spread of the early exponential area of the product accumulation curves.

Genotyping / Autom. threshold

❑ To activate the automatic calculation of the threshold value, click on the symbol.
Alternatively, you can call up the GENOTYPING / AUTOM. THRESHOLD menu command.

Whether you choose manual or automatic calculation, the resulting threshold value is updated and displayed synchronously in the THRESHOLD input field.

5.6.3 Specifying genotyping options

Special evaluation options are available for genotyping.

❑ Click on in the toolbar to open the GENOTYPING OPTIONS window.

Alternatively, you can call up the GENOTYPING / GENOTYPING OPTIONS menu command.

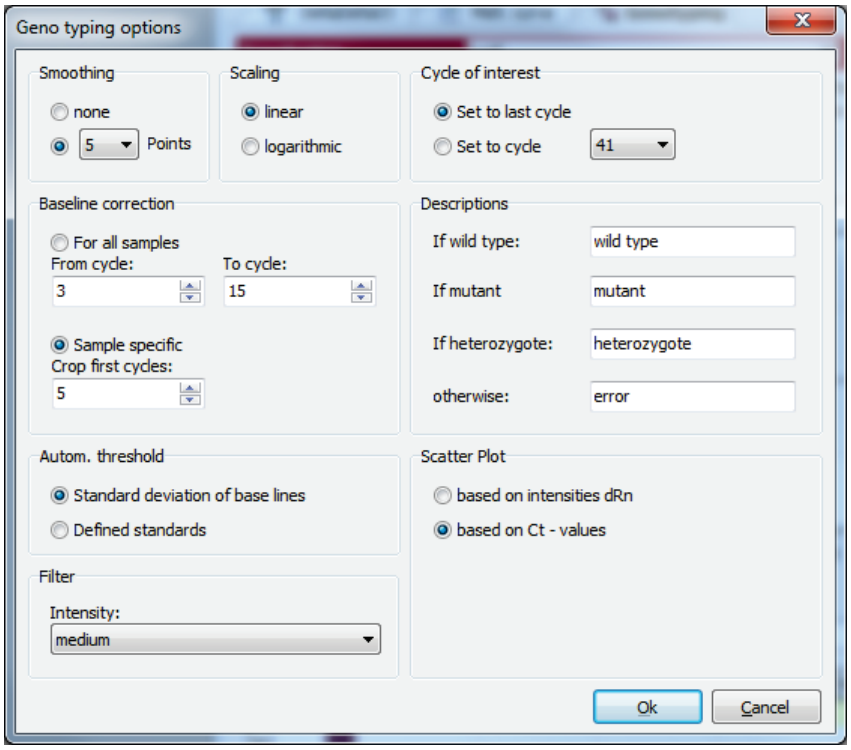


Fig. 82 GENOTYPING OPTIONS window for genotyping settings

Option	Description
CYCLE OF INTEREST	Select the cycle for evaluation. This can preferably be the last cycle (end point) but also any other cycle of the PCR run. The respective cycle can be selected from a list.
NAME	Input fields for own names for the wild type, mutant, heterozygote categories or otherwise
SCATTER PLOT	Generation of the scatter plot based on the fluorescence intensities of the analyzed cycle and/or the Ct values

5.6.4 Displaying the fluorescence curves, scatter plot and bar graph

The respective combination of wild type dye or mutant dye displayed is shown on the tabs in the bottom left corner of the area. The entry for the respective active combination on the tab is highlighted in white. The fluorescence data is displayed as a linear or logarithmic representation depending on the selected display option. For both view types a brief information is shown as soon as the mouse pointer is positioned on one of the curves.

1. Click on the button in the parameter bar.

   A selection window for the display options opens.

2. Select the SCALING LOGARITHMIC or LINEAR option.

   Click next to the selection window. The changes are applied.

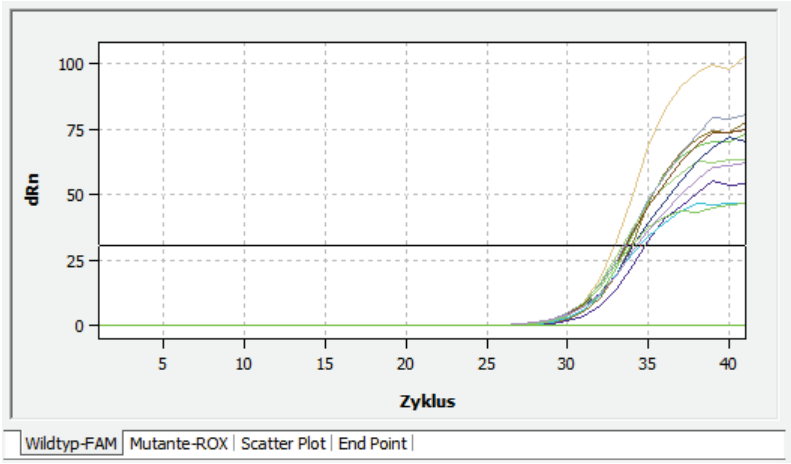


Fig.83 Linear representation of the fluorescence curve for genotyping

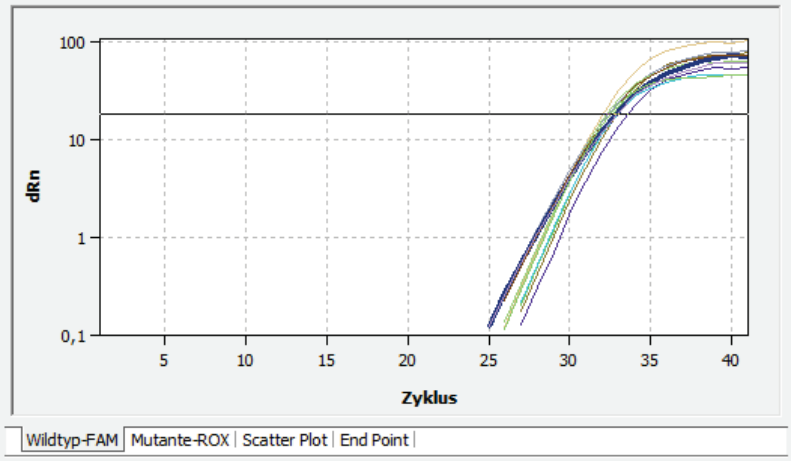


Fig.84 Logarithmic representation of the fluorescence curve with horizontal threshold line

To determine Ct values for the analysis a threshold value must be determined for the fluorescence curves. This can be automated but is also possible manually in the display area (→ "Parameter settings for genotyping" on page 85).

In addition to the fluorescence curves representation, the results can also be displayed as a scatter plot or bar graph. This can be selected via the respective tabs below the display area.

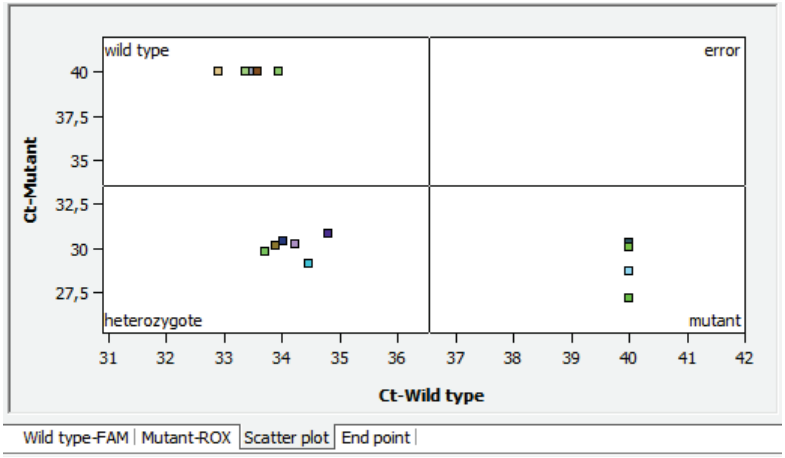


Fig. 85 Scatter plot for genotyping

The scatter plot is divided into four quadrants for mutant, heterozygote, wild type and error. The samples are assigned to one of the quadrants based on the

measured relative fluorescence or the Ct values of the two dyes. The respective cutoff value for the sample assignment is represented by two black lines in the scatter plot view. To change the position of the lines and thus change the cutoff value, select the lines and keep the left mouse button pressed. Alternatively, the respective cutoff value for mutant and wild type can also be entered in the respective fields in the selection area (→ "Parameter settings for genotyping" on page 85).

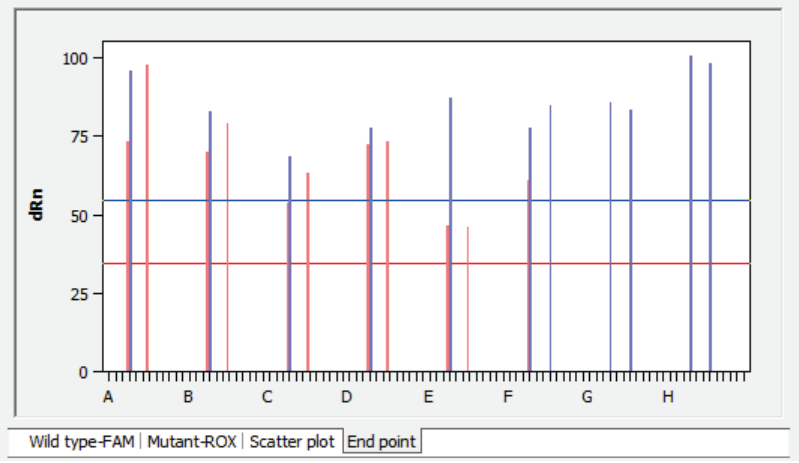


Fig. 86 Bar graph for genotyping

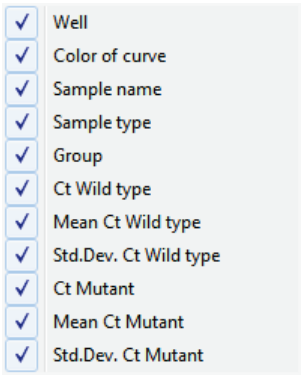
The bar graph shows the measured relative fluorescences as bars. The x-axis is scaled from A to H based on the rows of the block, i.e. the first twelve samples correspond to positions A1 to A12 in the block, the next twelve samples correspond to positions B1 to B12 etc. The cutoff value is set by moving the red or blue horizontal line up or down with the left mouse button pressed. Alternatively, the respective cutoff value for mutant and wild type can also be entered in the respective fields in the selection area of this view (→ "Parameter settings for genotyping" on page 85).

The cutoff values are thresholds after which the question whether a sample shows a reaction is answered with "Yes" (see REACTION WILD TYPE and REACTION MUTANT table columns).

5.6.5 Display of the values for the genotyping evaluation

The sample table for the genotyping combines all data and corresponding measurements for the samples. The columns shown in the sample table differ depending on the tab selected in the display area. The table for the fluorescence curves provides a summary which includes the measuring data of both dyes. If the fluorescence intensity at the end point is evaluated, the sample table for the scatter plot is the same as the one for the bar graph. The data summarized in the sample table for the fluorescence curves, however, partly differ from the data in the scatter plot or bar graph evaluation. The selection of columns displayed can be defined by the user. Right-click on a column header to display one of the following selection fields:

Fluorescence curves



Scatter plot or bar graph

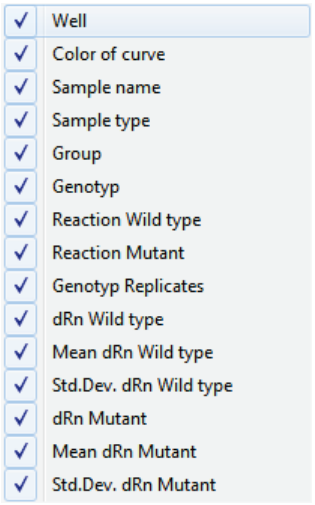


Fig. 87 Context menu for displaying the values in the sample table for genotyping

Column	Meaning
WELL	Position of sample*
COLOR OF CURVE	Each sample is automatically assigned an unchangeable color which is used to display the corresponding fluorescence curve
SAMPLE NAME	Name of sample*
SAMPLE TYPE	Type of sample*
GROUP	Assignment of the sample to an experimental group*
CT WILD TYPE	Ct value of wild type
MEAN CT WILD TYPE	Mean Ct value of replicates of the wild type
STD.DEV. CT WILD TYPE	Standard deviation of the Ct values between replicates of the wild type
CT MUTANT	Ct value of mutant
MEAN CT MUTANT	Mean Ct value of replicates of the mutant
STD.DEV. CT MUTANT	Standard deviation of the Ct values between replicates of the mutant
.	
GENOTYPE	Assign the sample according to wild type, mutant, heterozygote or error
REACTION WILD TYPE	Yes or no, depending on the end point fluorescence or Ct value
REACTION MUTANT	Yes or no, depending on the end point fluorescence or Ct value
GENOTYPE REPLICATES	Assign replicates according to wild type, mutant, heterozygote or error ("?" symbol)
DRN WILD TYPE	Standardized fluorescence intensity of the wild type reaction
MEAN DRN WILD TYPE	Standardized fluorescence intensity between replicates of the wild type reaction
STD.DEV. DRN WILD TYPE	Standard deviation of the standardized fluorescence intensity between replicates of the wild type reaction
DRN MUTANT	Standardized fluorescence intensity of the mutant reaction

MEAN DRN MUTANT	Standardized fluorescence intensity between replicates of the mutant reaction
STD.DEV. DRN MUTANT	Standard deviation of the standardized fluorescence intensity between replicates of the mutant reaction

Individual columns can be shown or hidden by selection or deselection. The layout of the columns can also be freely modified. By dragging a column heading with the left mouse button depressed columns can be swapped. The display of the results in the table can thus be adjusted as desired.

5.6.6 Deleting a genotyping

An evaluation that is no longer required can be removed.

1. Activate the evaluation by selecting its name in the evaluation list of the method tab.

Genotyping / Delete Genotyping

2. Click on in the toolbar.
   Alternatively, call up the GENOTYPING / DELETE GENOTYPING menu command.

   The evaluation is removed.

6 Functions in EXTRAS MENU

6.1 Device initialization

The device initialization sets the device to the original state. A device initialization is only required after an error has occurred.

Extras / Device initialization

❑ Call up the EXTRAS / DEVICE INITIALIZATION command.

6.2 Opening the lid (qTOWERonly)

The opening and closing functions of the qTOWER are motorized.

Extras / Open / close lid

❑ Call up the EXTRAS / OPEN/CLOSE LID command.

6.3 Exchanging the block (qTOWERonly)

To exchange the block, the thermoblock must be moved up. This movement must be software-controlled.

Extras / Change block

❑ Call up the EXTRAS / CHANGE BLOCK command.

6.4 Editing color modules

After inserting the color modules into the device's probe the color modules must be specified in the software.

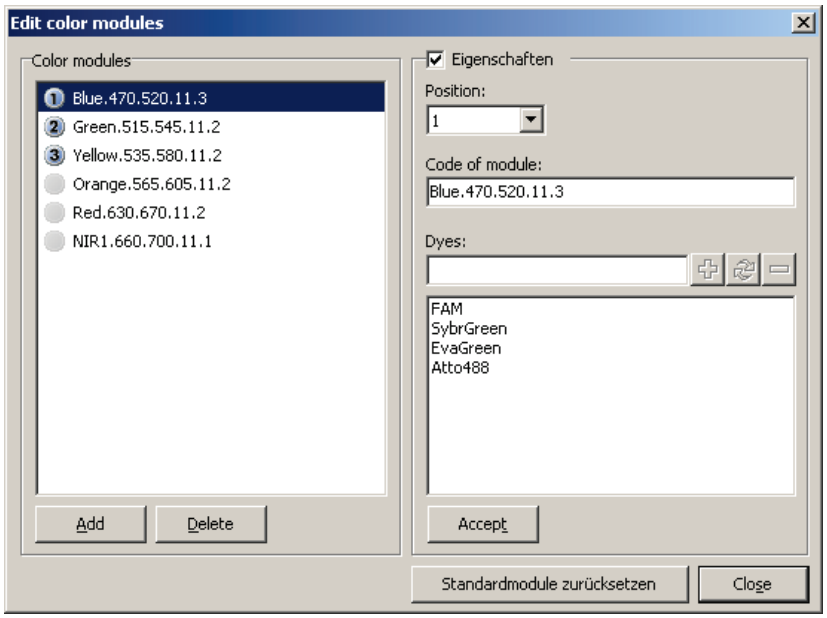


Fig. 88 Edit color modules window

Extras / Edit color modules

1. Call up the EXTRAS / EDIT COLOR MODULES command.

   A window with the same name opens.

2. The available color modules for your device are displayed on the left.

3. From the list, select the module you have installed in the device, activate the PROPERTIES checkbox and select the position on which the module is mounted in the device. If necessary, you can also add dye names if these have not yet been added to the list. Click on [ACCEPT].
4. Repeat this for each color module you have installed.
5. If you wish to define a new color module that is not included in the list of available color modules, click [ADD]. A new color module with the description COLOR.000.000.00.0 is created. You can define the properties on the right side of the dialog.
6. To delete a color module from the list, mark the module with the cursor and click on [DELETE].
7. To change the properties of a color module, proceed as follows:
   - Activate the PROPERTIES checkbox.
   - In the POSITION list select the position of the color module on the carrier in the fluorescence probe.
   - Enter the code noted on the color module in the CODE OF MODULE entry field.
   - Enter the dye that is detected with the color module.
     Click on [+].
     The dye is added to the list below.
     You can remove a dye by marking it in the list and clicking on [-].
   - Click [ACCEPT] to assign the properties to the marked color module.

Note:
A dye can only be assigned to one module. If it is to be measured with a different module, it first has to be removed from the first module.

6.5 Connecting the device to the PC

Extras / Device identification

The software qPCRsoft automatically recognizes which instrument is connected and whether it is switched on or not. The instrument may also be switched on and off during running qPCRsoft. Whether an active connection is established will be indicated in the lower left corner of the status line.

- ❑ If a connection cannot be obtained in between 30 seconds you can use the EXTRAS / DEVICE identification in order to solve the problem.

6.6 General settings in the qPCRsoft software

Extras / Options

General settings for the qPCRsoft program can be made in the OPTIONS window.

Note:
For most of the settings in the OPTIONS window you need to be logged into qPCRsoft as an administrator.

1. Select the EXTRAS / OPTIONS menu command to open the window of the same name.
2. Make the following settings on the tabs:

Tab	Function
GENERAL	Define that the last measurement results are saved automatically. (→ "Opening an automatically saved project" on page 15)
DATA FORMAT	Select decimal separator for the data export.
LANGUAGE	Select language of the qPCRsoft program interface.
MEASUREMENT	SENSITIVITY Set the basic sensitivity for optical measurements. MEAS. REP. COLOR COMP. Enter the measurement repetitions for recording the color compensation. SHOW NEGATIVE VALUES RESULTING FROM COLOR COMPENSATION If activated, negative values are also displayed in the sequence of the color compensation, otherwise the output is "0".
ANALYSIS	You can enter a factor for the quantitative evaluations (QUANT FACTOR), for the melting curve analysis (MELTING FACTOR) and for the genotyping (GENOTYPING FACTOR) in the list fields. This factor will be used for the automatic calculation of the threshold.
DEVICE	Enables you to define a file name into which the device communication data will be written. The recorded data is used for error diagnosis (→ Appendix)
FILE	If activated, the qPCRsoft program starts and the file opens if you select a file of this type in the file explorer of the operating system.
USER MANAGEMENT	Activate or deactivate the user management. If the user management is deactivated, no login prompt will appear at the program start. The functions for setting up the user management and for signing projects will not be available.

7 Working with the user management

Note on the general data security

Due to the encryption used, reading and changing project, template, evaluation and communication files generated by qPCRsoft is only possible with qPCRsoft.

7.1 User groups

The user management enables you to assign users to three different user groups with different rights:

Administrator

- Has unlimited rights for all program functions
- Can create, delete, block and unblock users and assign rights to them
- Can change his own password and those of the other users
- Can deactivate the user management in EXTRAS/OPTIONS/USER MANAGEMENT

User with general rights

- Can change his own password if allowed by the administrator
- Has the same rights as the administrator but with the following limitations:
  - Cannot create or edit users
  - Can only overwrite data after a confirmation prompt
  - Cannot create and edit color modules
  - No access to the MEASUREMENT, ANALYSIS, DEVICE, FILE and USER MANAGEMENT tabs in EXTRAS/OPTIONS

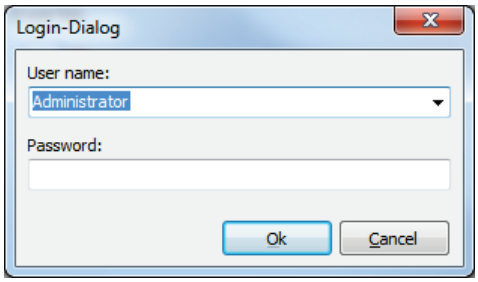
User with limited rights

- Can change his own password if allowed by the administrator
- Has the same rights as a user with general rights but with the following limitations:
  - FILE / NEW menu command is blocked
  - Cannot save project templates
  - Can open prepared project templates and start measurements with them, but cannot edit the template (exception: GENERAL tab)
  - SCAN / EDIT COLOR COMPENSATION is blocked
  - Can import prepared evaluations but cannot change the settings in the evaluation
  - Cannot access the EXTRAS/OPTIONS menu command

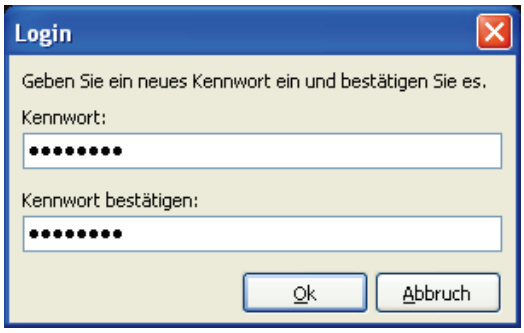
Setting up an administrator

After the program has been installed, an administrator **must** be set up and an administrator password defined.

1. Start qPCRsoft.
2. Click [OK] in the LOGIN DIALOG without entering a password.



3. Define the administrator password in the LOGIN window.



4. Set up the user accounts.
   If no user management is desired, deactivate it in Extras / Options / User management.

7.2 Managing user profiles

User profiles can only be set up by a user with administrator rights. Users may change their password if allowed by the administrator.

7.2.1 Adding a user profile

Edit / User management

1. Click on the EDIT / USER MANAGEMENT menu command to open the USER PROFILES window.
2. Click [ADD].
3. Enter the following data in the USER PROFILE window:

Option	Description
GENERAL tab	
USER NAME	Login name at program start
FULL NAME	Real name (optional)
DESCRIPTION	Further details (optional)
USER GROUP	Assign user group with the respective rights
PASSWORD tab	

PASSWORD	Enter password
CONFIRM PASSWORD	Repeat password
USER MUST CHANGE PASSWORD WITH NEW LOGIN	If activated, the user must change his password after the first login.
USER MAY CHANGE PASSWORD	The administrator allows the user to change his own password.
NO PASSWORD TIME OUT	Password is valid for an unlimited time. If deactivated, the expiry date must be specified.
USER IS DEACTIVATED	User profile has been locked by the administrator or because the wrong password was entered three times.
USER IS LOCKED	User profile has been locked by an administrator.

7.2.2 Editing a user profile / changing a password

1. Click on the EDIT / USER MANAGEMENT menu command to open the USER PROFILES window.
2. Mark the user profile in the list.
3. Click [EDIT].
4. Change the data in the USER PROFILE window.

Note:
If changing the password is allowed in the profile, users with general/limited rights can open their profiles in USER / USER MANAGEMENT and change their passwords. They do not have access to other settings.

7.2.3 Deleting a user profile

1. Click on the EDIT / USER MANAGEMENT menu command to open the USER PROFILES window.
2. Mark the user profile in the list.
3. Click [DELETE].

The user profile is deleted.

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